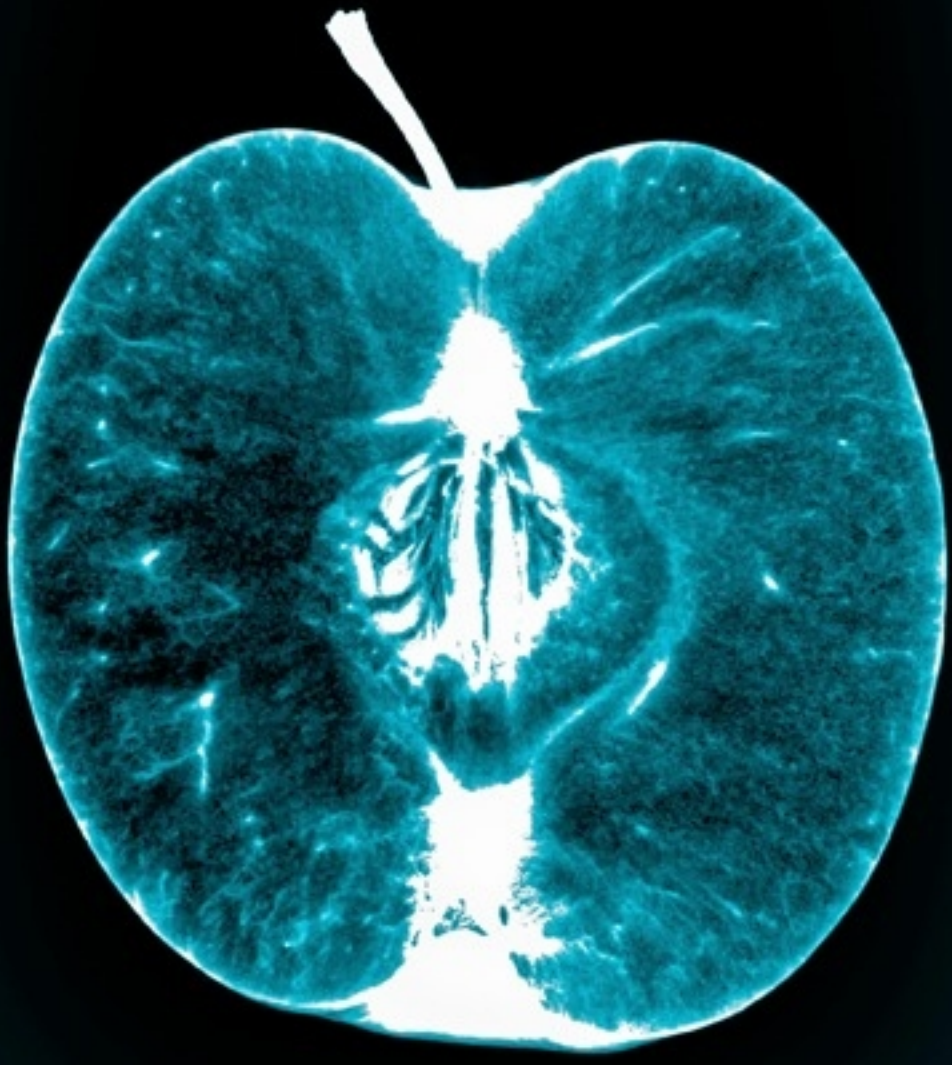
Cell

Volume 161
Number 1

March 26, 2015

www.cell.com



Biology of Food

In This Issue

Cell

"Tell me what you eat, and I will tell what you are." This quote, made famous by Brillat-Savarin in his seminal essay about gastronomy, summarizes the central role that food has played in the history of mankind. Food catalyzes reunion, celebration, and social identification. Conversely, the pursuit of food is also a driver of migrations and wars. Malnourishment still plagues underdeveloped areas across the globe, and the technological advancements needed to ensure the resources to feed a growing global population remain unmet goals. Paradoxically, overabundances of foodstuffs and dietary changes in the other areas of the contemporary world have produced changes in human biology that have triggered national health crises. The multifaceted ways in which food factors into biological, social, and political issues seem to only be getting more complex. This special issue, The Biology of Food, explores the science behind food, nutrition, and metabolism. Like any great menu, it offers plenty of options that we hope will first tantalize and then satisfy each individual's palate. The making of this issue has relied upon a special ingredient: the involvement of many dedicated authors and reviewers across all fields of biology, and we would like to thank them for their time, effort, and unique insights. We invite you to join us at the table. Bon appétit!

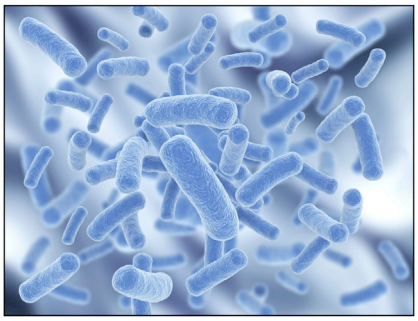
Ingredients for a Great Meal

Chefs and scientists share the desire to experiment, combine "ingredients" with precision, create, and perfect techniques to achieve outstanding results. In the Commentary that opens this issue (pp. 1–4), Pia Sörensen and Michael Brenner deconstruct molecular gastronomy, unraveling the biophysics behind the multitude of shapes, textures, and flavors that ingredients, as simple as an egg, can adopt. Benjamin Wolfe and Rachel Dutton delve deep into the world of fermentation—a microbial process that humans have harnessed for preservation and flavor development for over a thousand years. In this Review (pp. 49–56), they elaborate on how studying microbes that produce different qualities in cheese rind or kimchi provides opportunities to dissect mechanisms and general principles of microbial community formation.

Providing food security to an increasingly larger global population bathes food production in a sobering light. The expanding gap between demand and yield of primary foodstuffs highlights the potential for food shortages by 2050. In his Review (pp. 56–66), Stephen Long discusses emerging approaches that can improve crop photosynthetic efficiency to achieve a future with nutritious food for all.

A Balance of Nutrients and Flavor

Dietary balance is fundamental to health. What this "balance" represents and how it can be achieved are not so clear. A provocative series of Essays underscores research and technological breakthroughs needed to understand the complex effects of nutrition in human physiology and to combat malnourishment as well as overnutrition. Charles Zuker (Essay, pp. 9–11) discusses how the brain is wired to detect energy-rich food sources. Danny Ben-Zvi and Douglas Melton (Essay, pp. 12–17) take technology one step further, envisioning in vitro human stem-cell-derived digestive systems populated with gut microbiota to model disease and study nutrition. Complementing this Essay, the development of endocrine cells in the gastrointestinal system, and their roles in digestion and nutrient homeostasis, is beautifully illustrated in the Snapshot by Patrick McGrath and Jim Wells (pp. 176). In the final Essay, Stephen Simpson and his colleagues (pp. 18–23) present a different approach to the problem of dietary balance, framed from the perspective of nutritional ecology, in which integration of the interactive effects of multiple nutrients is key.



If dietary balance is indeed crucial for a healthy life, why do so many people prefer chocolate over broccoli? How do we make conscious decisions about what to eat? On pp. 24–35, Charles Spence provides a Perspective on the complex multisensory interactions that give rise to the flavor experiences we all know and love. He highlights that our perception of food relies on the integration of cues from all of the human senses and reveals how chefs and the food industry are taking the latest scientific findings on-board in their food design.

Matching Diet to Environment

For organisms to coordinate their growth and development with nutrient availability, they must be able to sense nutrient levels in their environment. David Sabatini and colleagues approach this question on the cellular level. In their Review (pp. 67), they discuss how these sensing mechanisms reflect the nutrient requirements of specific species and how they have adapted to support the emergence of multicellularity in eukaryotes. At the organismal level, a network of biological pacemakers known as the circadian clock directs and maintains proper rhythms in endocrine and metabolic pathways

required for organism homeostasis. On pp. 84–92, Gad Asher and Paolo Sassone-Corsi review the biological basis for clock adaptation to environmental changes, such as daily light-dark cycles and rhythmic food intake. Nutritional challenges reprogram the clock, while time-specific food intake deeply impacts physiology, calling for attention on the beneficial effects of chrono-nutrition.

Not only does an individual's environment influence their own metabolism, but in some cases the environment experienced by their parents also contributes to their risk of metabolic disease. Rebecca Simmons and Oliver Rando's Review (pp. 93–105) gathers evidence for the effects of parental diet on the offspring's metabolic phenotype in mammals and provides a current survey of mechanisms underlying these effects. As organisms develop and grow, they acquire companions for a lifetime. The microbiota, the collection of all microbes in symbiotic tête-à-tête with a host, is assembled early in life. Jeff Gordon and colleagues (Perspective, pp. 36–48) introduce the concept that microbiota immaturity is causally related to the pathogenesis of undernutrition and its lingering sequelae. They make a case for integrating this knowledge into efforts to develop new food preparations that sustain a healthy microbiota in children and mothers grappling with undernutrition. Understanding the configuration of microbiota in children representing diverse geographic backgrounds, cultural traditions, and states of health will be also required to assess the effect of the changes in eating practices brought about by globalization.

On the flip side, reduced food intake whilst avoiding malnutrition can ameliorate the effects of aging and aging-related diseases in model organisms and mammals. The effects of dietary restriction in longevity and healthspan are the focus of Luigi Fontana and Linda Partridge's Review (pp. 106–118).

Deconstructing Obesity

The global rise in the prevalence of obesity and co-morbidities such as type 2 diabetes, cardiovascular ailments, and cancer now represents a major public health concern. While the pathophysiology of such diseases is complex and multifactorial, the biological response to unbalanced consumption of palatable food and reduced energy expenditure is at the core of the problem. In "Hunger Genes: Pathways to Obesity" (Review, pp. 119–132), Agatha A. van der Klaauw and Sadaf Farooqi outline the genetics underlying the variability of the metabolic response to food in humans. The genetic framework will be valuable for the identification of effective mechanisms to prevent and treat obesity in susceptible individuals. On pp. 133–145 (Review), Joel Elmquist and his colleagues tackle obesity from the neuroscience perspective. They provide an up-to-date view on the neural circuits controlling energy balance and glucose homeostasis and their potential for pharmacological and surgical interventions. At last, Jonathan Brestoff and David Artis bridge the gap between immunology and obesity, discussing the emerging concepts in immune regulation of metabolism (Review, pp. 146–160). These three reviews offer a multidisciplinary understanding of metabolic diseases.

The special issue wraps up with two very special treats. On pp. 157–168, Nobel laureates Mike Brown and Joe Goldstein look at the past, present, and future of cholesterol research in the review "A Century of Cholesterol and Coronaries: From Plaques to Genes to Statins". Finally, Zhu Chen, Juleen Zierath, C. Ronald Kahn, Bruce Spiegelman, Stephen O'Rahilly, and Jens Brüning, all active leaders in the metabolism field, provide valuable solutions at different levels to stop the obesity epidemic and to improve health and well-being of people (pp. 173–174, Voices).

We hope that this special issue will connect researchers working in fields with different flavors and inspire scientists to pose new questions and pursue answers to the many unsolved mysteries behind the biology of food.

João Monteiro

Cell

Biophysics of Molecular Gastronomy

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http://dx.doi.org/10.1016/j.cell.2015.03.002

Chefs and scientists exploring biophysical processes have given rise to molecular gastronomy. In this Commentary, we describe how a scientific understanding of recipes and techniques facilitates the development of new textures and expands the flavor palette. The new dishes that result engage our senses in unexpected ways.

Molecular gastronomy's beginnings can be traced to the 1970s and an emerging curiosity about the scientific aspects of the foods we eat. As Nicolas Kurti, Professor of Physics at Oxford, famously stated, "It is a sad reflection on our civilization that while we can and do measure the temperature in the atmosphere of Venus, we do not know what goes on inside our soufflés" (McGee, 1984). Acting upon this sentiment and in collaboration with the physical chemist Hervé This, Kurti began to convene regular gatherings in Erice, Italy, bringing together professional chefs and scientists—among them, Pierre Gilles de Gennes, the Nobel prize winning physicist who popularized the study of physics of soft materials, as well as Harold McGee, who wrote the remarkable treatise "On Food and Cooking" (McGee, 1984).

This questioning of the biophysical basis of foods coincided with a burgeoning movement in the culinary world, spurred largely by the chef Ferran Adrià, who aimed to use emerging knowledge about the science behind recipes to rethink and reengineer foods and create new textures and tastes. Part of his program involved developing more precise methods for controlling cooking protocols, such as immersion heaters, rotovaps, and centrifuges. When these are applied to foods, a panoply of rich transitions are uncovered, leading to new textures and tastes.

The simplest example of creating new textures is the common task of cooking an egg. Chefs routinely cook eggs in boiling water, but an immersion heater allows cooking them at any desired temperature and observing sensitive changes in the texture that have been otherwise impossible to note. Which chef was the first to carry out precise temperature-controlled cooking on an egg is unclear, but a remarkable process was discovered: between 60°C and 70°C, critical biophysical transitions occur in chicken eggs; this is the range in which the different proteins within the yolk and white unfold. Strikingly, differences of less than 1°C lead to significant textural and structural changes. Indeed, a well-trained chef can predict the temperature of the water bath within a half a degree based on the texture of the egg, and eggs cooked even just a couple degrees apart have very different culinary applications (Figure 1).

At its core, the biophysical transitions that happen when cooking an egg are the same ones that biology regulates against: the unfolding and subsequent aggregation of proteins. Textures arise because denaturing proteins gradually expose previously buried fragments, enabling crosslinks to form between different proteins so that the protein mixture becomes a gel. As the temperature increases, more of the proteins are exposed, and the crosslinks become stronger and more numerous. This is due to both the continued unfolding of individual proteins and the ensuing unfolding of more stable ones. Microscopically, the unfolded proteins bond in complex patterns, and the resulting macroscopic texture arises from the nature of these arrangements. Thus, manipulating the crosslinked matrix by precisely adjusting the cooking temperature allows the control of macroscopic texture.

Several scientific concepts describe the macroscopic aspects of texture and how they relate to the underlying microscopic arrangement. One is quantified by the elastic modulus, which measures the resistance of the material to deformation. Roughly speaking, the elastic modulus $E = U_{\text{interaction}}/\ell^3$, where $U_{\text{interaction}}$ is the binding energy between the crosslinks, and ℓ is the typical distance between the crosslinks. The material thus becomes harder to deform when the binding energy or density of the crosslinks increases. Another aspect of texture is plasticity, which occurs when crosslinks easily remodel; a material with high plasticity will not recover its original shape when deformed. Yet another concept is the yield stress, which is the minimal stress that can be applied to the material for the crosslinks to break and the material to fracture. Soft condensed matter science has given a qualitative understanding of how these macroscopic properties are related to the microstructure of materials. But how they apply in detail to the intricate suite of textural transitions in an egg is not well understood; we do not know how the microscopic structure of the egg's crosslinked interior changes as a function of temperature nor how the different parts of egg proteins contribute to the final texture. Importantly, we also don't know which physical properties correspond to the mouthfeel of a food.

These questions are not just academic: for example, if the biophysics of the transitions in an egg were better understood, it might be possible to redesign the egg to have different properties as it is heated. One could imagine mixing different concentrations of the protein constituting egg whites to manipulate the textural transitions, or mixing egg proteins taken from different organisms with distinct denaturation and aggregation properties. Moreover, tuning individual proteins to prescribed macroscopic

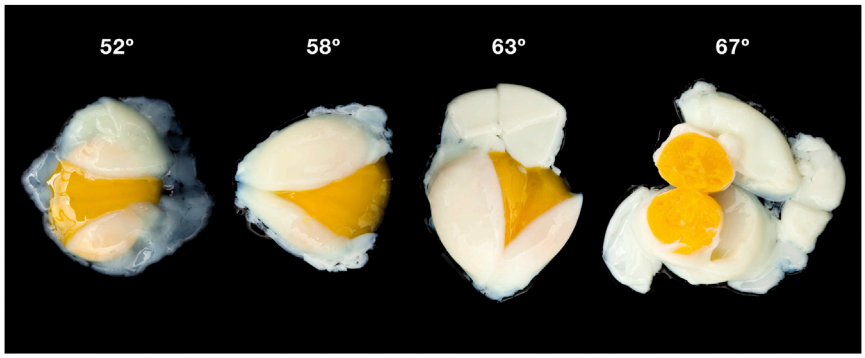

Figure 1. Eggs Cooked at Different Temperatures in a Temperature-Controlled Water Bath
Even small differences in temperature result in significantly different textures. Image courtesy of *Modernist Cuisine*.

properties could have utility well beyond cooking.

Another aspect of Adrià's culinary revolution involved introducing new ingredients. One such set of ingredients are molecules called hydrocolloids, which can be used to create new textures in the form of different types of emulsions, foams, and gels, all hallmark components of the modernist kitchen. A particular explosion occurred with gelling agents, a subgroup within this category, which mostly consists of carbohydrates. Whereas a century ago, the major gelling agent in Western-style kitchens was gelatin derived from animal collagen, today's chefs routinely use gelling agents derived from a diverse range of organisms that allow control of texture and properties in new regimes. These ingredients include methylcellulose, xanthan gum, agar, and gellan, each having distinct biophysical properties that differ from the standard gelling agents. For example, whereas gelatin gels between 4°C and 35°C, methylcellulose gels between 50°C and 90°C. This property, allowing creation of a hot gel, led to Adrià's invention of hot ice cream, which has the same texture and rheology as ordinary ice cream but only when served at high enough temperatures. Similarly, spherification, one of Adrià's signature dishes and characteristic of molecular gastronomy, also makes use of a gelling agent: alginate, a polymer found in sea weed, which forms crosslinks with calcium ions, resulting in a thin gel that encapsulates a sphere of liquid flavor (Figure 2A). Another example is gellan, derived from the bacterium *Pseudomonas elodea*, which has both a high melting temperature and also a low yield stress. This material enabled Heston Blumenthal to create a famous dish, hot and cold tea (Blumenthal, 2009), in which a glass of tea is divided into two halves: the left half is served cold, whereas the right half is served hot. Gellan strengthens the gel sufficiently to separate the two halves of the tea cup but breaks up immediately upon pouring. The textures of the two teas are identical, allowing the consumer to simultaneously taste hot and cold tea on different sides of the tongue.

To date, chefs have capitalized on the tremendous diversity of gelling agents produced by organisms in the natural world to create a wide range of foods in new parameter regimes. There is much room for further creativity, with increased understanding of why the different gelling agents work as they do. For example, one could imagine engineering organisms to produce gelling agents with distinct properties.

Thus far we have discussed culinary manipulations of food texture; the second major aspect of cooking affected by the culinary revolution is flavor. Flavor arises from the combination of several sensory experiences—most notably, the thousands of taste receptors on our tongues and the olfactory receptors in the upper nasal passages. While the number of tastes that can be experienced by humans are limited to the well-known five—sweet, salty, sour, bitter, and umami—we can sense orders of magnitudes more smells. Flavor compounds tend to be small molecules, and in the case of olfaction they are also volatile, which allows them to be transported from the food to the nasal passage. While many flavor molecules exist as preformed compounds in the cooking ingredient and are released when the tissues of foods are damaged, as is the case with the pungent molecules in mustard seeds, others are formed during the cooking process through the application of heat, mechanical force, or other procedures. This is the case for many fruity aromas, which are created when tissue damage releases enzymes that react with molecules in fruit cells to produce esters.

There are three noteworthy methods for producing small flavor molecules in modern haute cuisine. The first is to capture and concentrate them. Modernist chefs use a variety of techniques to extract, isolate, and concentrate flavor and aroma molecules, many of which draw on methods and equipment from biology and chemistry laboratories. These range from filtering to centrifugation to vacuum infusions. For example, Nathan Myhrvold uses centrifugation as a way to isolate flavorful carotene butter from carrot juice and laboratory sieves and agar filtering to concentrate purees and soups (Myhrvold et al., 2011). One of the most popular pieces of equipment for isolating and concentrating flavor is the rotovap. Long a stalwart of chemistry laboratories but introduced in the last decade into high-end cooking, rotovaps allow gentle, low-heat separation of certain molecules based on their volatility. Thus, compounds that would easily boil off or decompose at standard boiling temperature can be captured and served as part of a dish. For example, Joan Roca, of the restaurant Can Roca in Spain, uses a rotovap to capture the flavor molecules of eucalyptus leaves and citrus peel, both consisting of molecules that easily vaporize and disappear; the distillates are then used as a base for sorbets and other cold desserts (Figure 2B). Roca also uses the same technique to make a gel from distilled forest soil, which he then serves with an oyster, creating a completely novel flavor combination that would be impossible to achieve without the rotovap.

A second method for producing flavor is creating small flavor molecules through chemical reactions. Aspects of this practice have clearly been a part of cooking long before the advent of molecular

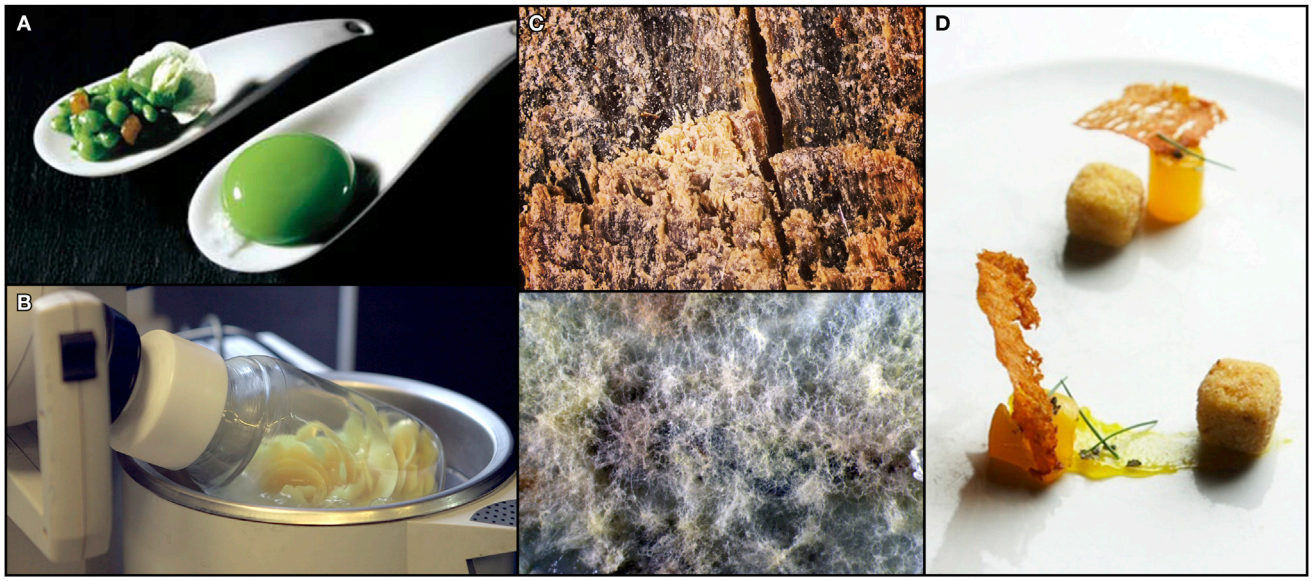

Figure 2. Examples of Dishes from *Modernist Cuisine*
(A) Ferran Adrià's spherified pea ravioli. Image courtesy of Ferran Adrià.
(B) Joan Roca's rotovapped citrus peel. Image courtesy of Blua Producers.
(C) Close-ups of David Chang's pork bushi, a novel take on the Japanese fermented fish known as katsuobushi. Image courtesy of Momofuku.
(D) Wylie Dufresne's deconstructed dish, eggs benedict. Image courtesy of Takahiko Marumoto.

gastronomy—simply heating or cutting into foods can initiate chemical reactions—but recent developments in techniques and equipment have not only brought advances in this field, but have also suggested further potential for discovery. One example is the diversity of flavors produced by caramellization and Maillard reactions. Caramellization denotes the decomposition of sugars at high heat, whereas Maillard reactions result from complex reactions initiated by amino acids reacting with sugars. Both reactions are highly temperature and pH sensitive and must be carefully controlled not to result in strong and bitter flavors. However, if correctly manipulated, these reactions contribute to the complex flavors of diverse foods ranging from steak to maple syrup to champagne. Modernist chefs found ways to enhance these effects using pressure cookers, which not only speed up flavor production, but also produce more intense flavors. Though Maillard and caramellization reactions create marvelous results even with a relatively modest knowledge of the underlying chemistry, both could potentially be deconstructed and controlled even more carefully, with the opportunity of isolating novel flavors from the plethora of flavor molecules resulting from these reactions.

A third method of producing new flavors in foods is fermentation, which relies on microbes breaking down large molecules in vegetable and animal products, such as proteins, carbohydrates, and fats, into an array of much smaller molecules. Fermentation is an age-old method of producing intense, complex, and incredibly diverse flavors: coffee, soy sauce, cheese, chocolate, wine, and vinegar—the contemplation of almost any list of fermented foods is a telling illustration of the powerful relationship between fermentation and flavor. In many cases, flavor production is the primary purpose of the fermentation reaction, such as in soy sauce and traditional fermented fish sauces. But in other cases, the flavor molecules are delicious byproducts of what might be taken to be the primary goal of the process. For instance, in wine production, where the preserving and intoxicating properties of ethanol from glucose drive the fermentation, it is the myriad enzymatic byproducts that create the complex and nuanced flavors of wine.

A relatively recent trend in haute cuisine is the experimentation with fermentation reactions to create new foods. David Chang, for example, works with unconventional combinations of foods and microbes to create new recipes, such as pomegranate seeds with lactic acid bacteria, commonly used in dairy fermentations, or locally grown farro with *Aspergillus oryzae*, a mold traditionally used in Asian cuisines for miso and sake production (Figure 2C). The design and characterization of such novel fermentation processes are still in their infancy but hold much potential as a field for further discovery.

We hope that we've succeeded in bringing across the message that the biophysical properties of molecules underlying cooking provide much interesting fodder for scientific inquiry. The culinary revolution has, however, pushed this notion further, and the design of new dishes has itself become a highly experimental enterprise. In the process of optimizing the incorporation of new ingredients and equipment into the kitchen, chefs experiment in ways similar to scientists. Failure of a given idea gives rise to new ones, eventually leading to creations that might not even be related to the original idea. Heston Blumenthal described this process in the context of creating the recipe for hot and cold tea as involving "endless trials" and

"infinitesimal precision" (Blumenthal, 2009). This is an attitude to cooking that sticks to this day and is apparent in many aspects of molecular gastronomy. For example, a common theme in modernist kitchens is the frequent and complete overturn of the menu preceded by intense periods of research and experimentation. Ferran Adrià initiated this trend at his famed restaurant elBulli, which closed for 6 months of the year while his team withdrew to a workshop in order to exclusively focus on the creation of next year's menu. Interestingly, the emphasis on culinary experimentation not only expanded cooking into the realm of science, but it also allowed it to take on characteristics from the arts. With the realization that new ingredients and equipment do more than simply offer an opportunity to tweak traditional recipes, chefs began exploring the creation of uniquely novel dishes. These creations play on the faculties of all senses, including notions of emotions and memories evoked by tastes and aromas, our expectations of food, and the element of surprise as a dish turns out to be something different from what it appears to be. As an example, a common theme in the modernist kitchen is the deconstructed dish, which separates the components of a familiar recipe into individual pieces with novel and imaginative textures. Wylie Dufresne's take on eggs benedict, which consists of deep-fried hollandaise sauce served with concentrated egg yolk shaped into columns, is an example of this approach (Figure 2D). Another common theme is playing with the diner's expectations by presenting foods that look different from what they are. Accordingly, Joan Roca famously serves an ice cream dish that is indistinguishable in shape from that of a cigar, and José Andrés of the restaurants Jaleo and minibar uses spherification to make eggs in which the white has been replaced by a rich solution of parmesan cream.

In summary, the past two decades have seen an emergence of striking complementarity between the methods, ideas, and culture of the culinary world and basic science. The chef's goal of transforming the properties of cells and proteins into another form is complementary to scientist's efforts to understand them. There is much left to be learned at this intersection.

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Food for the Brain

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http://dx.doi.org/10.1016/j.cell.2015.03.016

Diet is a major issue facing humanity. To combat malnourishment and diseases associated with overnutrition, both research and technological breakthroughs are needed.

It is 2015, the world population is approaching 7.5 billion, and there are nearly a billion malnourished people (Food and Agriculture Organization of the United Nations). At the same time, developed countries are experiencing catastrophic increases in metabolic syndrome, obesity, diabetes, and cardiovascular disease, all likely related to diet.

Can science help us develop better ways to feed ourselves? This, of course, is a complex question with many potential answers—from innovations in agricultural sciences and crop production, to changes in livestock farming, to implementing and enforcing broad changes in the sustainable use of land and marine resources. Much has been written and debated on each of these topics. I believe that real change will require breakthrough disruptive technologies and transformational changes in policy—mere incremental improvements are unlikely to change the food system, or our eating habits, in a timeframe that matches the challenge.

In this brief Essay, I will consider three attractive opportunities in my own field that may help provide solutions to these challenges: (1) understanding our brain circuits controlling appetite for sweet; (2) developing ways of producing intrinsically palatable, novel protein-rich nutrients in a low cost, self-sustainable, renewable, high-capacity platform; and (3) elucidating the links between our diet, the microbiome, gut-brain circuits, and metabolism. Ultimately, it may be possible to prevent disease through our diet.

Our Love for Sugar

Sugar (originally from sugarcane) was first produced in New Guinea some 10,000 years ago (Smith, 1995), and brought to Europe as crystalized "honey powder" from the Indian Territories by the armies of Alexander the Great, and later, as "sweet salt," by crusaders returning from the Holy Land. By 1800 the average American consumed approximately 7 pounds of sugar a year (Elliott, 1917). Today, the average American consumes over 100 pounds of added sugar annually (USDA, 2014) (Figure 1), and according to the Centers for Disease Control, more than 1 in 4 people in the US have metabolic syndrome (Ervin, 2009). By point of comparison, Americans consume ~50 pounds of beef annually.

Our craving for sugar is likely rooted in brain circuits dedicated to reward the recognition of high-energy food sources—a mechanism essential for animals in the wild, and most certainly critical in our own evolutionary trajectory, but terribly misused and abused by humans today (in essence by hijacking this pathway for our own pursuit of pleasure) (Lutter and Nestler, 2009, Volkow et al., 2011, Nieh et al., 2015).

Sweet compounds are detected by specific taste receptor cells on our tongue and palate epithelium; sweet-sensing cells send hardwired, appetitive, consummatory signals to our brain (Yarmolinsky et al., 2009). These circuits permit the identification of energy-rich food sources, and their association with a highly positive (i.e., rewarding) brain state. Remarkably, animals can develop a strong preference for sugars completely independently of the taste system, so that even in the absence of a functional taste signaling pathway, they still acquire a strong drive to consume sugar (de Araujo et al., 2008; Sclafani and Ackroff, 2015). Defining the sugar-selective elements of this circuit may provide valuable strategies to modify our sugar-craving eating habits and help combat obesity and associated metabolic disorders. For example, by identifying the sensors that detect the (taste-independent) sweet stimulus and transfer that information to the brain it may be possible to modulate our "hunger" for sugar.

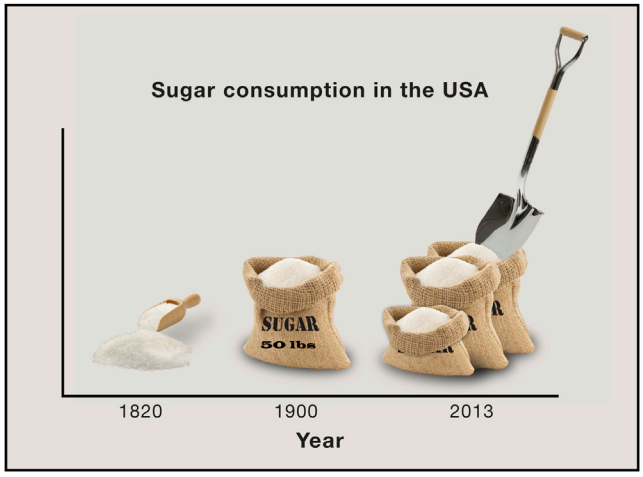

Figure 1. Increase in Sugar Consumption in the USA
Americans consumed approximately 7 pounds of sugar in 1820, 50 pounds in 1900, and over 100 pounds in 2013.

Protein Food

Proteins are regularly produced in significant amounts both for pharmaceutical and

industrial uses, with current technologies being adequate for many "niche" needs (e.g., industrial enzymes and protein-based therapeutics) (Wurm, 2004). There are a number of efforts at producing plant-derived meat substitutes and artificial meat (for example Beyond Meat http://beyondmeat.com/, Modern Meadow http://modernmeadow.com/, Impossible Foods http://impossiblefoods.com/, Cultured Beef http://culturedbeef.net/; see links for details); these are creative approaches that provide high-value, technologically intense alternatives to animal meat products. However, the kind of technology that addresses world needs would have to be simple, sustainable, easily transferable, inexpensive, and with a low carbon footprint. It takes thousands of liters of water, and tremendous amount of energy to produce just 100 g of beef protein (this includes the water and fuel needed to grow the animal feed, to process it and to transport it (Mekonnen and Hoekstra, 2012). Of note, over 60% of the grain produced in the USA is used to feed livestock (Cassidy et al., 2013). Not surprisingly, producing 1 calorie of animal protein requires 10 (or more) times the amount of fossil fuel required to produce 1 calorie of plant protein.

I believe we need to dramatically reduce our consumption of animal meat, but also harness the power of synthetic biology toward the production of alternative protein-rich food sources (for example by producing protein that may have inherently beneficial properties, and formulating them as an inexpensive, appetizing food product). However, this will require technology that scales-up biosynthetic efficiency by at least 2–3 orders of magnitude. Best-of-class current technologies yield about 0.2–1.0 g of protein per liter (Zhu, 2012); to make this proposal a viable strategy we would need to efficiently produce at least 100-fold more, and do so in a cost effective way. In this context, it would also be highly preferable to have the protein product itself exhibit intrinsic sensory properties that make it highly palatable (for example in texture and taste). Given that taste receptors can be selectively activated by amino acids, peptides, and proteins (Nelson et al., 2002), this might be an attainable goal.

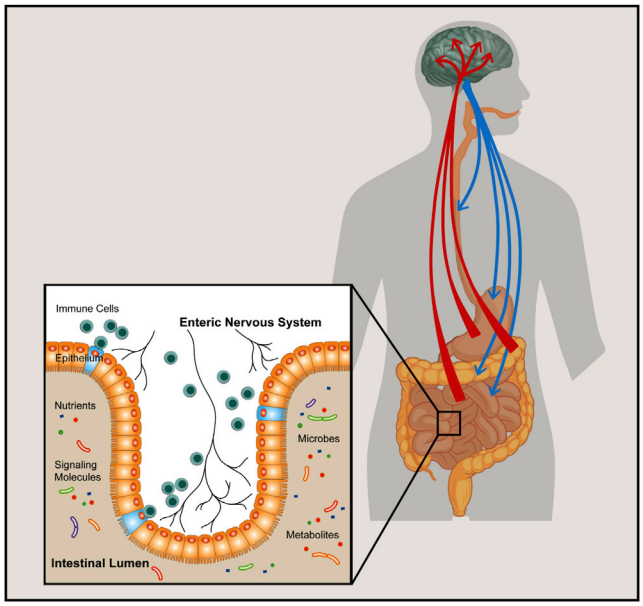

Figure 2. Interplay between the Gut, Microbiome, and Brain
The gut-brain axis is a bidirectional neural signaling system connecting the gastrointestinal system (and other internal organs) to the brain. For illustration purposes, nerve fibers are shown freely contacting the gut epithelia, and no other tissue is shown on the basal side of the gut epithelia (i.e., lamina propia).

The Microbiome-Gut-Brain Axis

Our diet modifies the microbiome, and the microbiome modifies our diet. Although not generally presented as such, this statement underscores the link between the microbiome and human physiology (Sekirov et al., 2010). Indeed, it is now evident that gut microbes impact what the human host is capable of extracting from its diet, from nutrients to bioactive signaling molecules. Understanding the biological interactions between our diet and our intestinal microflora provides an immense opportunity to improve the nutritional value of food and human health. We have many examples, including recent studies in which gut microbiota are transferred between mice with vastly different metabolic states, and in doing so changing the new host's metabolism (Vijay-Kumar et al., 2010). In this brief perspective, however, I want to highlight a related, but very distinctive link to the gut: the gut-brain axis (Figure 2).

Our gut is innervated by some 300 million neurons (a mouse brain has ~100 million neurons) that monitor and inform the brain about our internal physiological and metabolic state (Furness, 2012). I envisage this "information highway" between our gut and our brain as offering unprecedented "access" to brain centers involved in metabolic, physiological, cognitive, and emotional states. Unraveling the role of these gut-brain circuits could change the way we think about food, nutrition, and human physiology.

In this issue of *Cell*, leading researchers review and confront a wide range of questions dealing with food, physiology, and human health—from advances in crop production, to exploiting the physical and chemical properties of food ingredients to create new sensory experiences in flavor (taste, odor, texture, temperature, and presentation), to new insights into mother-child metabolic imprinting, to transformative advances in the control of cholesterol metabolism. This is an exciting time in science. This collection of papers provides a window into recent advances and future opportunities.

ACKNOWLEDGMENTS

The author thanks Robert Barretto and Laura Rickman for help with editing, and Tania Gutierrez, Tom Maniatis, and members of the Zuker lab for helpful comments. Charles Zuker was a scientific co-founder of Senomyx and Elcelyx Therapeutics.

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Cell

Leading Edge
Essay

Modeling Human Nutrition Using Human Embryonic Stem Cells

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http://dx.doi.org/10.1016/j.cell.2015.02.039

Nutrition presents unanswered scientific questions of high public health importance. We envision model systems composed of interacting gastrointestinal and metabolic tissues derived from human embryonic stem cells, populated by gut microbiota. The culture will be embedded in 3D scaffolds, creating a controlled experimental system that enables tissue sampling and imaging.

Introduction

In the 19th century, important advances in physiology arose from an unusual relationship between the physician William Beaumont and his patient Alexis St. Martin. St. Martin received a shotgun wound that left him with a sizeable opening in his stomach that did not close. Beaumont availed himself of St. Martin's unique condition and conducted some of the first digestion experiments. For 10 years he continued this research combining in vivo and in vitro approaches. For example, by tying food to a string and inserting it through the hole in St. Martin's stomach and retrieving it later, Beaumont was able to retrieve the food and observe digestion in real time. He also removed gastric juice from St. Martin's stomach watched it digest food in a test tube, providing new information on the mechanical and chemical nature of digestion.

Obviously, the scientific community has come a long way since then, with new methods to study physiology and nutrition using cell lines, model organisms, and endoscopic views of the human digestive system. Nutritional scientists studying microbiology, physiology, neurobiology, immunology, epidemiology, genetics, and behavior have made substantial progress in understanding the biology of food.

Nonetheless, we have yet to comprehensively answer some major nutritional questions of wide public interest. For instance, is being vegetarian beneficial to one's health, and if so, how? Is organically farmed food healthier or only more expensive? How does the introduction of new crops and cooking methods affect our health? Can nutrition reduce risk for autoimmune diseases? Is it the high carbohydrate, high fat content, or both, of western diets that leads to obesity and metabolic syndrome? (Antico et al., 2012; *Nature* Editors, 2014; Feinman et al., 2014; Key et al., 2006)? Even in cases with conclusive epidemiologic data, the underlying mechanisms that link nutrition and pathology are not entirely clear: how does obesity lead to development of insulin resistance? How does a Mediterranean diet prevent cardiovascular disease (Estruch et al., 2013; Qatanani and Lazar, 2007; Ye et al., 2012)? These unanswered questions lend themselves to nutritional myths and inconclusive recommendations that have become all too commonplace in the daily news. As a result, the public is easily confused by opposing views supported by scientific evidence (Malik and Hu, 2007; Matarese and Pories, 2014; Willett and Stampfer, 2013).

Nutritional questions may be easy to pose, but they are difficult to answer for reasons we outline below. Here, we propose a stem-cell-centric point of view and suggest that human stem cell models of digestive processes could be a powerful experimental system to study the physiology of digestion and nutrition in the future.

Challenges in Nutritional Science

Many discoveries in physiology have been made possible by the use of model organisms, including fish, rodents, and flies. Advanced experimental tools were developed to study these organisms, and they will continue to provide invaluable data for many years to come. However, since each species evolved to respond to distinct nutritional challenges, their responses to food differ; indeed, our diet and lifestyle are strikingly different from those of model organisms. Moreover, most model organisms have limited genetic and environmental variability. On the other hand, primary and immortalized human cell lines are easy to manipulate, are amenable to imaging and screening, and provide highly reproducible results. Yet, immortalized cell lines differ metabolically from cells in vivo and are limited in their ability to teach us about the function of organs and the intra and inter-organ signaling.

Digestion is a complex process performed physically and chemically by the gastrointestinal (GI) organs and exocrine glands together with gut bacteria. Food is sensed in the gut by enteroendocrine cells that prepare the body for the incoming meal: For example, the hormone Cck regulates the release of pancreatic enzymes, Pyy signals to neurons that regulate satiety and behavior, Glp1 sensitizes insulin-secreting cells in anticipation for the increase in blood glucose (Psichas et al., 2015). The immune system sustains a symbiotic relationship with gut bacteria necessary for digestion (Round and Mazmanian, 2009), and the enteric and nervous systems regulate gut motility, behavior, and blood circulation through chemical and physical sensing of food. Metabolites themselves act as signaling molecules, capable of binding nuclear receptors such as the PPAR proteins. All cells consume metabolites and change their cell biology in accordance with food availability, linking nutrition to every process in the body and making nutrition a Gordian knot.

Variability between individuals must be taken into account. Not only do we have different eating habits and taste preferences, but we also vary in our ability to digest and metabolize food. Metabolic variability of individuals is largely unexplored and may be affected by numerous factors, including culture, psychology, genetics, gut microbiome composition, epigenetics, and neuroendocrine regulation.

Given these difficulties, how might one begin to systematically tackle the question of how nutrition affects health? Although we cannot literally create a window into the human gut to obtain Beaumont's view, researchers today would benefit from direct access to the human gut. Fortunately new technologies are converging in a manner that may allow us to recreate a functional GI system in vitro. Our ability to generate organoids and tissues from human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs) continues to make rapid progress. We may soon be able to assemble them into a miniature GI system in a dish or even on a chip. This would allow for the manipulation and analysis of digestion in a reproducible, accurate, and large-scale manner, and facilitate exploration of the effects of individual genetic and microbial diversity. Here we discuss how these pieces might come together to aid the science of nutrition.

Building a Functional Gastrointestinal System In Vitro

Intestinal organoids are miniature, three dimensional, star-shaped versions of the full intestine consisting of a single epithelial layer with crypts and villi. Stem cells at the base of the crypt can be isolated from human and mouse primary cultures, and can generate multiple cell types, such as pit cells, enteroendocrine cells, and mucus glands facing a miniature lumen. Remarkably, these organoids can be grown for long periods of time, and have been used by many labs now to study in vitro digestive enzymatic activity, development of the gut, bacterial infection, and structural aspects of the crypt-villi unit (Sato and Clevers, 2013). Impressive progress has been recently made toward differentiating hESCs into intestinal and gastric organoids (McCracken et al., 2014; Spence et al., 2011) opening a new range of research avenues on understanding human gut biology and disease through the use of hESCs and iPSCs.

Other cell types can also be differentiated from hESCs. Insulin-secreting β-cells can be grown from hESCs in clusters comparable in size to an islet of Langerhans (Pagliuca et al., 2014). Similarly, a protocol for deriving glucagon-secreting α-cells has been established (Rezania et al., 2011). This raises the possibility of building a human stem-cell-derived islet (SC-islet) containing all the pancreatic endocrine cells types. Many labs are developing methods to differentiate hESCs into other cell types such as adipocytes (Cuaranta-Monroy et al., 2014), hepatocytes (Takebe et al., 2013), and myocytes (Salani et al., 2012) and find appropriate culture conditions to keep cells functional for extended periods of time (Sachs and Clevers, 2014). It is likely that we will be able to generate functional organoids and cell types such as SC-liver and SC-gut for most metabolic organs.

The ability to genetically modify stem cells that are used to make the tissues and organs will be enormously valuable in determining which genes function in which cells to affect phenotype. Advances in gene editing by TALEN and CRISPR technologies make it possible to test both loss and gain of function for specific genes and organs.

A complementary advance has come from bioengineering, making "organs-on-a-chip." The chips are microfluidic devices, populated by cell cultures organized and perfused such that they resemble tissue and organ biology more than the classic tissue culture in a dish. The technology aims to preserve the advantages of cell culture, such as accessibility, control, and reproducibility in an environment that recapitulates in vivo physiology.

In recent years we have observed a tantalizing increase in the sophistication of organs-on-a-chip, and an emergence of a vibrant bioengineering community supporting these efforts. We are still learning how to improve upon these first generation models, and there are several challenges to overcome. Manufacturing is not entirely robust, and requires expertise of in-house engineers. More importantly, we are missing the functional equivalent of blood: a medium that will support the culture of many cell types and transport biomolecules between different organs. This bottleneck must be relieved before a modular, multi-organ system can be incorporated to study digestion and nutrition, or any other multi-organ process. Nonetheless, researchers have been able to generate increasingly complex functional models of angiogenesis, blood brain barrier, cardiovascular system, nutrient absorption, and liver drug metabolism (Bhatia and Ingber, 2014).

In addition, maturation of the SC-organs remains a critical challenge for the success of any stem-cell-derived model. Intestinal SC-gut organoids have improved their function after transplantation under the kidney capsule of a recipient mouse (Watson et al., 2014). It is hypothesized that vascularization is critical for this process, so proper maturation of organs may require co-culture with vascular endothelial cells, which can also be derived from stem cells (van der Meer et al., 2013).

If these challenges can be met, it should be possible in the future to generate an in vitro system composed of several human ES- or iPS-derived cell types and organoids that will absorb food from the luminal side and be connected basally by fluid to liver, pancreas, muscle, and/or adipose SC-derived tissue, delivering hormones and nutrients from one tissue to the next (Figure 1). This would circumvent problems arising from using immortal cell lines to generate organoids, as these display altered cellular metabolism and generally do not give rise to in vivo 3D structures such as a liver lobule or an islet of Langerhans. Clean human primary cultures are difficult to obtain, especially from many tissues at the same time. In vitro differentiated cells overcome these problems and can reproduce specific genetic backgrounds which can be useful for personalized medicine or simply to introduce human genetic variability into research.

Building reliable in vitro systems capable of recapitulating digestion is a monumental task requiring the collaborative efforts of labs from several fields in biology, chemistry, and engineering. Some key parts of the system are missing and integration of the stem-cell-derived

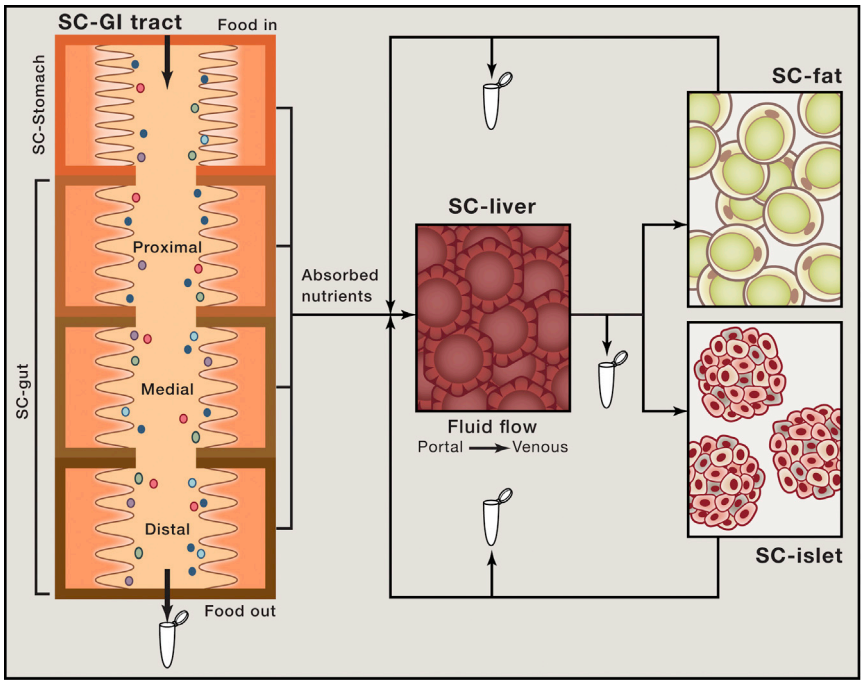

Figure 1. Schematic Depiction of a Human Stem-Cell Based in Vitro System to Model Digestion and Nutrition

Food travels through different sections of the SC derived gastrointestinal segments populated by gut microbiota. Absorbed nutrients, hormones, basally secreted proteins, and lipids are transported to an SC-liver, which recapitulates to portal-venous axis in liver lobule. Fluids are then fed to other metabolic organs such as SC-islet of Langerhans and SC-WAT. Potentially other SC-tissues can be added. Secreted hormones and protein-lipid complexes are fed back to close the loop (feeding of intestinal segments not shown for clarity of presentation). At each point, a fluid or food sample can be drawn, and SC-tissues are available for biopsies. Important technical consideration such as waste collection and perfusion channels are not shown in this schematic depiction.

tissues in a bioengineered system will be challenging. But considering the progress in the last decade in stem cell and bioengineering fields, there is good reason to believe that creating a physiological, human-based model of digestion is possible.

Incorporating Gut Microbes and Food In Vitro

An in vitro human digestive system as described above would only partially model digestive physiology. Two other important factors that need to be incorporated are food itself and the gut microbiota.

The human gut is populated by trillions of bacteria from hundreds or thousands of strains. Gut bacteria effectively form a functional organ, composed of many microbial strains that interact with the human host and perform various metabolic functions. The recognition of the importance of microbiota-host interactions is certainly not new; however, technological advancements in DNA sequencing and other methods have led to a booming increase in our knowledge on this topic (Li et al., 2014). As a result, digestion is now understood to be a joint human-microbial process. The food we eat nourishes not only our organs, but also the microbial community that we host.

The challenge of maintaining a microbial ecosystem within the lumen of an in vitro system is daunting. We are just beginning to understand how to culture gut microbial strains in a dish (Goodman et al., 2011), but initial steps toward this aim have been made. An advanced model of a gut-on-a-chip, populated by the gut anaerobic bacterium *Lactobacillus rhamnosus* GG and human intestinal Caco-2 cells, has been developed. Co-culturing bacteria and cells may be easier on a chip due to the constant flow of nutrients and differential culture conditions on the luminal and apical side of the intestinal epithelia (Kim et al., 2012; Kim and Ingber, 2013). We do not yet know if in vitro systems can or should host a full complement of gut microbes, how such an ecosystem can be inoculated and sustained in culture, or what types of nutrients the bacteria will require to model digestion accurately.

In addition, the microbiota responds dynamically to diet, perhaps more so than the human gut. It changes with age and has tight interactions with its host through metabolites and the immune system. It is also highly variable between individuals and societies, representing a major source of metabolic variability (Parfrey and Knight, 2012). In vitro modeling of the gut microbiome is still at early stages, and some bacterial strains are not easily cultured in the lab. Experimenting with this system will teach us about human-microbiota interactions and explore how metabolic variability relates to microbiome composition.

In terms of food, the duodenum, jejunum, ileum, and colon have different digestive functions and encounter food at different digestive stages. In vitro systems should be designed such that hESC-derived intestinal segments form separate chambers (Figure 1) that pass food and chyme at various stages of digestion through the lumen. Tailored luminal culture conditions and chip mechanics will be required to support the introduction of food and provide appropriate conditions for gut microbiota to flourish.

Nutritional Data In Vitro and In Vivo

Nutrition science is a vast field integrating biochemical, physiological, bioinformatic, and cultural data. Mathematical modeling and big data analysis are needed, as in gut microbiota research, which includes sequencing of multi-strain samples, building statistical models of gut microbiota, and overlay of metabolic networks with microbial enzymes. Breakthroughs in microbiome research have been made possible through collaborations between microbiologists, biochemists, and computational biologists using system biology approaches (Human Microbiome Project Consortium, 2012).

Metabolomics, the high-throughput mass spectrometry quantification of metabolites in cells, organs, and body fluids,

benefits from the collaborative efforts of biochemists and computational biologists, and the data analysis tools are comparable to those of sequencing and gene expression (Melamud et al., 2010). In the future, many more studies will likely use metabolite profiling to describe the physiology of human subjects in health and disease, leading to discovery of novel roles for metabolites and refining the known metabolic networks at the cell and organism-microbiota levels (Ryan et al., 2014).

Similarly, nutrigenetics and nutrigenomics advance our understanding of human metabolic variability using human genetics and microbiome sequencing. A classic example is the discovery that mutations in the *ldlr* gene cause familial hyperlipidemia. More recent efforts to relate genetics, diet, and disease include a series of biochemical and genome-wide association studies aimed at exploring connections between genetic polymorphisms, red meat, and vegetable consumption to the development of colorectal cancer (Figueiredo et al., 2014). The Segal and Elinav labs take a different approach to understanding human metabolic variability by measuring glucose levels and food intake over the course of a week and sequencing the gut microbiome of individuals. The results are fed to a machine learning algorithm that generates a personalized diet aimed at reducing glucose spikes following meals (www.personalnutrition.org).

Many tools and approaches developed today will be instrumental for analyzing the hESC based in vitro metabolic system we envision. The various parameters influencing metabolism can be well controlled and, compared to in vivo studies, it should be possible to collect high quality, reproducible data. For example, we will be able to sample fluids containing absorbed metabolites from different positions in the in vitro gut and understand how they are affected by gut microbiota, a sudden change in diet, antibiotics, or fasting and feeding. Biopsies from the in vitro system could teach us about the proteomic and transcriptomic profile of the liver in response to feeding, with high temporal resolution. Alternatively, biopsies and fluid samples may be obtained from the system over a long period of time, without worrying about the compliance of the patient to the prescribed diet, effects associated with the immune and nervous systems of the model organism, and other uncontrolled variables.

Live imaging is much easier in vitro than in vivo. Imaging metabolic or adaptive processes is limited today to simple in vitro cultures, or to snapshots from in vivo models. Using hESC reporter lines, one will be able to track the expression of specific genes, cell composition, secretion of factors, and changes in the morphology of human tissues.

In all, the inherent complexity of nutrition and digestion may be reduced and dissected with a hESC-based in vitro system. Access to high quality imaging, metabolomics, and genomics data will allow analysis of tractable mathematical models based on large data and testing of specific biological hypotheses without the inherent difficulties of immortal cell lines.

Applications and Limitations of a Human Stem-Cell-Based System

An in vitro, complex stem-cell-based system can fill the gap between an animal in vivo model and cell lines, but cannot model the full complexity of an in vivo scenario and is not as straightforward as a cell line or primary culture. However, it can complement existing models and human studies and create an opportunity to better address physiological and nutritional questions.

The ability to use iPSCs and genome editing technology has been already put to effective use for disease modeling (Rashid et al., 2010; Wang et al., 2014). A stem-cell-based system can be used to study nutritionally related diseases. For example, several genes have been associated with intestinal inflammatory diseases, but the role of only a handful of genes has been elucidated (Murthy et al., 2014). It is not known whether changes in the immune system, the intestine at large, or gut microbiota triggers disease in genetically susceptible patients. More generally, the ability to genetically modify stem cells that are used to make tissues and organs will be enormously valuable in determining which genes function in which cells to affect phenotype. The advances in gene modification in this regard are all in place, making it possible to test both loss and gain of function for specific genes.

An in vitro system has applications for screens of food additives or synbiotics. Currently, studies are done on cell lines, using mice or cohorts of humans, and are not practical for the challenge. For example, how would we test which bacterial strains are most efficient at digestion of complex carbohydrates? Numerous combinations of bacterial strains should be tested in several dietary conditions and human genetic backgrounds. Such experiments would require thousands of mice, but an in vitro system might be scaled up for the task. Similarly, a controlled system is ideal for studies on the uptake of food and drugs. The differences between model organisms and humans become crucial in studying drug metabolism, and an in vitro system might better model drug metabolism, improving the efficiency of drug development.

We envision through this system will address many puzzles in the physiology of digestion. We will be able to watch bacteria break down food and pass metabolites to enterocytes, measure the level of hormones as they are secreted, and watch the intestine and microbiota adapt to a change in diet. It should be possible to model the digestive track following bariatric surgeries and study the positive and negative effects of surgery on metabolism (Mingrone and Castagneto-Gissey, 2009). It will also permit modeling of fecal transplants in specific genetic backgrounds and diseases.

Ultimately, our hope is that this in vitro system will go beyond the physiology of digestion into the field of nutrition. The aforementioned unanswered questions in nutrition involve the long term effects of diet, but currently organs-on-chips have been limited to shorter time scales on the order of weeks. This limitation makes such systems less valuable for the study of long term processes such as chronic disease development. However, if indeed the in vitro system can sustain cultures for longer periods of time, one could follow the metabolic profile of tissues following long term exposure to a specific diet. One could capture the development of insulin resistance in a tissue as a function of diet in a system simple enough to isolate in vivo effects exerted by the immune and nervous system, but complex enough to capture micro-environmental effects, such as liver

zonation. Similarly, we could compare the long term effects of high carbohydrate versus high protein diets on two initially equivalent systems. These data will be instrumental in understanding the mechanisms underlying epidemiological and nutritional observations in human subjects (Qatanani and Lazar, 2007).

Nutritional questions that have been raised recently, such as the advantages in organic foods or the effects of artificial sweeteners, could be addressed directly. Mice studies are illuminating, but currently, there is not sufficient human data to draw conclusions (Gardner et al., 2012; Suez et al., 2014). By using a human-based system and data of high quality and resolution, we might be able to construct better full scale tests on human subjects, and understand the mechanisms by which sweeteners, both natural and artificial, affect our long-term risk to develop type 2 diabetes. We could feed a stem-cell-based system with organic versus non-organic food, and compare the metabolic effects on human tissues and microbiota in a controlled setting (Barański et al., 2014; *Nature* Editors, 2014), then propose better hypotheses and design new experiments on model organisms and human subjects. Ultimately, an in vitro system could support decisions made daily by clinical nutritionists.

There are, or course, inherent limitations to such an in vitro system. It does not recapitulate all aspects of organ physiology; for example, the nervous and immune systems may prove difficult to integrate into this system. The effects of nutrition on organs such as the heart will require an addition of a heart-on-a-chip or other compatible systems (Wang et al., 2014). An in vitro model will be smaller by 2–3 orders of magnitude than the human gut, and this physical difference limits the ability to model all physical and chemical processes. Finally, the engineering challenges in manufacturing and handling these systems may keep them from being widely applied.

Conclusions

The phrase ''an apple a day keeps the doctor away'' speaks to a link between nutrition and health. However, as a scientific community we have yet to answer comprehensively basic questions in the science of nutrition. Human nutrition is difficult to study because of its complexity, variability between individuals, and human's new foods and unique eating habits and physiology. Despite hurdles and bottlenecks, the generation of complex, stem-cell-based in vitro systems simulating physiological processes is just around the corner. We believe that the approach outlined here will allow us to begin to answer some basic, practical questions about how nutrition affects our immediate health, behavior, and the development of disease. Who is prone to gain weight and why? What is a healthy diet and how might this differ from one person to another? Nutrition scientists have good answers to some of these questions based on epidemiological data and controlled animal and human studies, but these answers lack mechanistic understanding and may not apply to specific individuals. Ultimately, we are faced with a staggeringly simple and vital question: what should we have for dinner?

ACKNOWLEDGMENTS

D.B.-Z. is supported by the Human Frontiers in Science post-doctoral fellowship. D.A.M. is an Investigator of the Howard Hughes Medical Institute. We thank Dr. Jonathan Schneidermann and Dr. Sagi Levy for critical reading of this manuscript.

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Cell

Leading Edge
Essay

Putting the Balance Back in Diet

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http://dx.doi.org/10.1016/j.cell.2015.02.033

The notion of dietary balance is fundamental to health yet is not captured by focusing on the intake of energy or single nutrients. Advances in nutritional geometry have begun to unravel and integrate the interactive effects of multiple nutrients on health, lifespan, aging, and reproduction.

Diet Balance Problem

One of the most important and prominent public health messages is to eat a healthy, balanced diet. But what does that mean? Balanced with respect to what—and when during the life course? What are the consequences of failing to achieve a balanced diet? These are fundamental questions that remain less well answered than is necessary to devise effective public health policy to combat the pandemic of obesity and metabolic disease (Simpson and Raubenheimer, 2012). Here, we show that advances from nutritional ecology are providing new ways to address these problems.

The classical approach to understanding diet balance has been painstakingly to derive individual estimates for required intakes of each of the dozens of macro- and micronutrients that are needed for health and wellbeing. Such "one variable at a time" (OVAT) approaches (Box et al., 1978) have provided the foundations of nutrition science. The evidence-base has been built from a combination of animal studies in which single constituents have been manipulated in experimental diets, epidemiological analysis of the associations between intakes of single nutrients and health outcomes in human populations, and single-variable clinical trials. These data have in turn informed national dietary guidelines with associated recommended daily intakes (RDIs) for micro- and macronutrients. Clinical practice, food labeling policies, and public health strategies have followed.

There is an abundant literature showing that fats, sugars, salt, vitamins, etc. contribute to health outcomes, but one consequence of taking a single-variable approach has been to promote adversarial debate between proponents of single-nutrient causes (or solutions) to diet-related health problems. This is nowhere better illustrated than in the long running debate over the roles of sugar and saturated fats in obesity and metabolic disease (Feinman, 2011; Willett, 2011). As a result, public confusion reigns—even (perhaps especially) among the well-educated populace—fuelled by commercial interests in the food sectors and the fad diet industry (Simpson and Raubenheimer, 2014).

The fundamental problem with OVAT approaches is that they fail to capture the multidimensional essence of nutrition (Ruohonen and Kettunen, 2004). It is axiomatic that diets are more than the sum of their components; they are combinations of foods, each comprising mixtures of nutrients and other constituents. Changing the concentration of one component in the diet can alter the character of the entire blend. In simple statistical terms, OVAT looks only at the main effects of single nutrients and does not account for the interactions between nutrients within diets—neither the non-independence of dietary constituents within mixtures nor the interactive effects of nutrients on health outcomes.

We need an approach that explicitly takes account of the interactions among nutrients within foods and diets and is able to define and quantify the consequences of different diet compositions on multiple measures of health across the life course. In this essay we illustrate such an approach, known as the geometric framework, which originated from the field of nutritional ecology (Raubenheimer et al., 2009). Nutritional geometry integrates not only multiple diet components, but also scales across molecules, cells, organs, organisms, populations, and ecosystems (Simpson and Raubenheimer, 2012). Starting with the ideas of nutrient-specific appetites and regulatory priorities, we introduce the concept of nutritional response landscapes using model organisms including *Drosophila* and mouse, and then discuss the application of nutritional geometry in humans.

Geometry of Nutrient-Specific Appetites

A fundamental requirement for considering the multilayer interactive effects of nutrients is to establish the extent to which the intakes of different nutrients are specifically regulated by the animal. In other words, are there so-called "nutrient-specific appetites" distinct from intake control merely based on total dietary energy or volume? Nutritional geometry provides a series of simple yet powerful concepts and experimental designs for addressing this question. One example has been to explore whether an animal has the capacity to regulate its intake of two nutrients simultaneously when challenged with different pairwise combinations of nutritionally complementary foods varying in their ratio and/or concentrations of the two focal nutrients. If animals converge upon the same ratio and amounts of the nutrients eaten

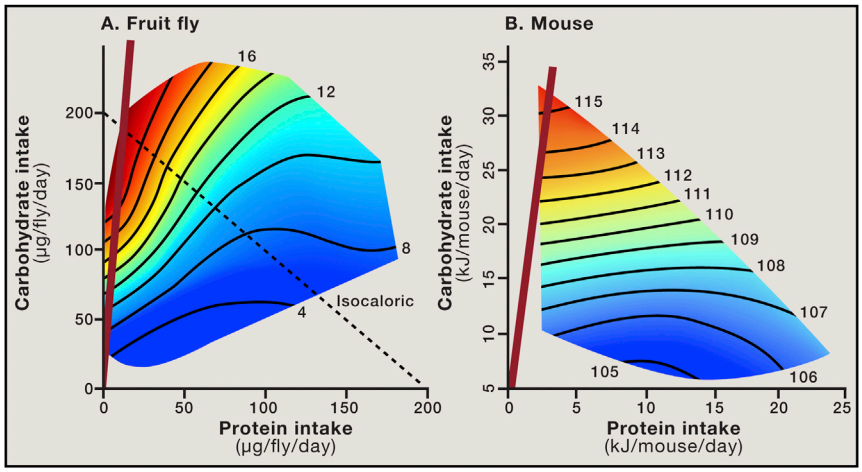

Figure 1. The Relationship between Protein and Carbohydrate Dietary Intake versus Lifespan in Flies and Mice

(A and B) Flies (Lee et al., 2008) (A) and mice (Solon-Biet et al., 2014) (B). In both cases, lifespan was maximized by diets with low ratios of protein to carbohydrate (red lines).

("intake target") across experimental food pairings, in each case ingesting the unique amount of each food required to do so on that particular pairing, it is then evident that the animal has separate regulatory systems controlling intake of the two nutrients. Similar types of experimental design have been used to show that organisms from acellular slime molds all the way to primates possess nutrient-specific appetite systems for macronutrients, such as proteins, carbohydrates, and fats, as well as for at least two micronutrients, sodium and calcium (Simpson and Raubenheimer, 2012). However, most micronutrients do not seem to be specifically regulated; rather, their intakes are maintained within healthy limits by a combination of correlation in foods with other regulated nutrients and non-specific mechanisms such as learned aversion to foods associated with development of a micronutrient deficiency, coupled with heightened attraction to novel foods (Simpson and Raubenheimer, 2012).

Having demonstrated that specific appetites exist for certain nutrients, the question arises as to how these are prioritized when the animal is restricted to a diet composition that does not allow the intake target to be reached for all regulated nutrients simultaneously. Under such circumstances, the animal must balance eating too little of some nutrients against overconsuming others relative to the intake target. Understanding how animals prioritize different nutrients under these circumstances is of considerable importance for appreciating or predicting the health impacts of shifts in diet (Lihoreau et al., 2014; Raubenheimer and Simpson, 1997). As a premise, we need first to be able to map nutritional response landscapes.

Mapping Nutritional Outcomes in *Drosophila melanogaster*

Drosophila provides a simple system for illustrating how to map the consequences of nutrition in multiple, potentially interacting nutrient and response dimensions. Lee et al. (2008) used nutritional geometry to disentangle the effects of calories from those of macronutrients in the context of increased lifespan upon caloric restriction (Curtis and de Cabo, 2013; Everitt et al., 2010; Mercken et al., 2012; Speakman and Mitchell, 2011) and also explored the basis for the frequently reported trade-off between aging and reproduction (Tatar, 2011). Flies offer several advantages for this type of analysis. First, their dietary calories come principally from two macronutrient sources—protein and carbohydrate (lipids, although essential, provide only a small caloric contribution)—thereby defining a two-dimensional nutrient space. Second, flies are small and short-lived, making large numbers of dietary treatments in a longevity study feasible.

In this study, flies were confined throughout their lifetime with ad libitum access to one of 28 diets, comprising seven protein to carbohydrate ratios (P:C), each at one of four total concentrations. Response landscapes for longevity and reproductive output were mapped onto an array of individual P:C intakes recorded for more than 1,000 flies, thereby allowing the consequences of nutrient and energy intakes to be visualized and analyzed. The results were striking (Figure 1A). Low-calorie intake per se was not associated with prolonged lifespan in *ad libitum*-fed flies; rather, lifespan was a function of the ratio of protein to carbohydrate ingested, declining as P:C increased. Second, lifespan and reproduction had differently shaped response landscapes with peaks in different places on the protein-carbohydrate intake plane—the diet composition that sustained longest life led to a lower intake of protein than needed to support maximal reproductive success. When allowed to compose their own diet by selecting among complementary food pairings, flies chose to mix a diet maximizing reproductive output rather than lifespan. Subsequent studies have shown that the trade-off between lifespan and reproduction is not obligatory or causal, but simply reflects differing nutritional optima for the two traits (Grandison et al., 2009; Tatar, 2011).

From Flies to Mice

A similar experiment has been conducted in mice (Solon-Biet et al., 2014). Here, the aim was to extend the use of nutritional geometry to quantify, *inter alia*, the impacts of macronutrients on food intake, body composition, lifespan, reproductive potential, cardio-metabolic health, immune status, mitochondrial function, gut microbiota, and nutrient signaling pathways. Nine hundred mice were confined from weaning with *ad libitum* access to one of 30 diets. These comprised ten protein to carbohydrate to fat ratios (P:C:F), which systematically sampled the 3D macronutrient mixture space, each ratio provided at one of three total energy densities by dilution with cellulose. Of the 30 diets, five that were very low (5%) in protein, high in fat, and low in energy density failed to sustain growth in young mice and were discontinued. Food intake was recorded throughout the experiment.

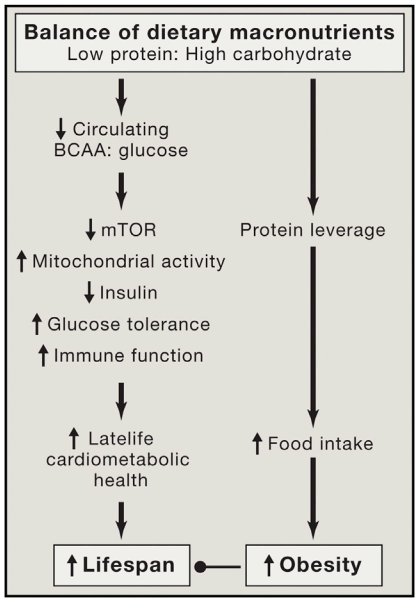

Figure 2. A Low Dietary Protein to Carbohydrate Ratio Has Counterposing Effects on Food Intake and Late Life Cardiometabolic Health

Mice, like other animals, possess separate macronutrient appetites (Sørensen et al., 2008), and when these were forced to compete by restricting animals to a single diet composition, total food intake was driven principally by protein, increasing as percent protein in the diet fell (consistent with compensatory feeding to stabilize protein intake). Compensatory feeding for carbohydrate was also apparent, with intake increasing as percent carbohydrate fell in the diet but to a somewhat lesser degree than for protein. Unlike protein and carbohydrate, however, the concentration of dietary fat had little influence over total food intake. Consequently, total food and energy intakes were maximal on diets combining low percent protein with high percent fat.

Energy intakes in turn corresponded to the body composition of mice, with adiposity increasing as a function of energy intake. Even though mice on low P:C diets were moderately adipose (although not to the extent of low-protein, high-fat fed mice), they lived longest (Figure 1B). Indeed, longevity mirrored the pattern seen in flies, being greatest on low P:C diets. Markers of metabolic health (insulin, glucose tolerance) and immune function at 15 months of age were consistent with the longevity data, being best on low P:C diets and worst on high-protein and high-fat diets (Le Couteur et al., 2014; Solon-Biet et al., 2014; Figure 2). By contrast, measures of reproductive potential in both males and females were highest on a higher-protein diet, consistent with results from flies.

There was no evidence for prolongation of lifespan on *ad libitum* diets that restricted calorie intake by reducing the energy density of the diet. The standard regime for restricting calorie intake that is well known to extend lifespan involves providing mice with a daily aliquot of food, which is soon eaten, leaving the animal deprived for the rest of the day (Curtis and de Cabo, 2013; Everitt et al., 2010). By inference, then, the results of Solon-Biet et al. (2014) imply that extension of lifespan with standard caloric restriction protocols may not entirely be secondary to reduction in calories; rather, other factors may contribute such as periods of fasting (Mattson et al., 2014), and reduction in protein intake that ensues once the mouse has eaten its daily food allocation.

A major conclusion from the geometric experiments on flies and mice is that the balance of macronutrients in the diet has a profound impact on food and energy intake, metabolic health, lifespan, immune function, and reproduction. The diet composition that best supports longevity is not the same as that which sustains maximal reproductive output or leanness. The question arises as to whether these conclusions apply to humans. The evidence suggests that they do.

Humans Behave Like Mice and Flies

For mice on diets differing in the ratio and concentrations of protein, carbohydrate, and fat, food intake was driven most strongly by the concentration of protein in the diet, but with a strong competing feedback emanating from signals associated with the specific appetite for carbohydrate. The data from population survey analyses (Austin et al., 2011; Austin and Krueger, 2013; Martinez-Cordero et al., 2012), compendia of controlled trials (Gosby et al., 2014), and detailed clinical studies involving foods formulated to disguise their macronutrient composition (Gosby et al., 2011) indicate that prioritization of protein intake may be even stronger in humans. Humans compensate for reduction in the available proportion of dietary energy contributed by protein by increasing food intake, and in so doing over-ingest fats and carbohydrates. Since the percentage of energy from protein in the diet is always smaller than that from fats and carbohydrates combined, compensatory adjustments in intake that redress relatively small deficits in protein "gear up" to relatively large excess of fats and carbohydrates, and thus energy intake overall—what we have termed "protein leverage" (Simpson and Raubenheimer, 2005). Studies have shown that total energy intake is, indeed, a negative function of percent protein in the diet across the range seen in all human populations measured to date with food sufficiency, namely 10%–25% protein of total energy. Above ca. 20%–25% protein the reduction in intake with rising percent protein becomes attenuated (Gosby et al., 2014), presumably because of increasingly strong opposing feedbacks arising from deficiency of other nutrients, notably carbohydrate, driving increased intake. At the other extreme, clinical trials using 5% protein (Martens et al., 2013; Martens et al., 2014a, b) failed to show increased energy intake relative to 15% protein diets, indicating that, as in mice and other animals, there is a lower limit to compensatory responses to dietary protein. Five percent protein approximates the composition of French fries from fast-food outlets, and is insufficient to maintain lean mass. Maintaining protein intake at adequate levels on such a diet would require ingesting an unfeasible quantity of food.

Gosby and colleagues (2011) showed that the 12% increase in *ad libitum* energy intake among subjects confined to a 10% protein diet relative to 15% or 25% protein diets was due to increased consumption of savory-flavored foods between meals. The seeking of savory cues is indicative of protein hunger, and is reflected in increased activity in brain regions associated with reward, such as the inferior orbitofrontal cortex and striatum (Griffioen-Roose et al., 2014). These results indicate that protein status influences gustatory pathways in a way that affects protein intake in humans. In insects, feedbacks onto gustatory

responses occur at the periphery, through direct modulation of taste receptors, as well as via learning of nutrient-specific cues (Simpson and Raubenheimer, 2012). The mediating nutrient signaling systems controlling protein appetite are thought to involve both circulating free amino acids and lean hormonal signals such as FGF 21 (Laeger et al., 2014).

Controlled, prospective experiments testing the effects of multiple diets, equivalent to those performed in animals, are not feasible in humans. Nevertheless, there is growing evidence from observational studies and quasi-interventional trials indicating that health and lifespan are influenced by the balance of macronutrients and can be best interpreted using nutritional geometry. In a systematic review of human dietary studies (Pedersen et al., 2013), it was concluded that long-term, high-protein, low-carbohydrate diets and increased mortality are associated. In addition, long-term, high-protein, high-fat and low-carbohydrate diets increased the risk of type 2 diabetes mellitus. Consistent with this notion, Fung and colleagues (Fung et al., 2010) reported that high-protein, low-carbohydrate diets were associated with increased mortality over 20–26 years in the Nurses' Health Study and the Health Professionals' Follow-up Study. Similar results linking low-carbohydrate, high-protein diets with increased mortality and/or cardiovascular disease have been reported in the Swedish Women's Health and Lifestyle cohort (Lagiou et al., 2012; Lagiou et al., 2007) and the Greek cohort of the European Prospective Investigation into Cancer and Nutrition (Trichopoulou et al., 2007). These studies have specifically reported the balance of two macronutrients, protein and carbohydrate, and consistently indicate that low-carbohydrate, high-protein diets increase mortality. Such conclusions are consistent with results in animals where the balance of macronutrients, rather than the intake amount of either, is a key determinant of lifespan, and that diets with high-carbohydrate and low-protein were associated with increased lifespan and improved cardiometabolic outcomes in late life (Lee et al., 2008; Solon-Biet et al., 2014). These conclusions are indirectly supported by associations between increased mortality and low-carbohydrate diets in humans (Noto et al., 2013) and a recent study showing increased mortality and cancer on high-protein diets (Levine et al., 2014).

In demonstrating that both high and low P:C diets have benefits and risks, these data clearly illustrate the importance of dietary balance. But a conundrum remains (Figure 2). Whereas a low P:C diet appears beneficial for longevity and late life health, protein leverage on such a diet tends to drive overconsumption of total energy and risk of obesity, thereby mitigating the health benefits of low-protein intake. Another consideration is that overweight in humans might be associated with poor outcomes if caused by low-protein, high-fat diets, but better outcomes when low-protein, high-carbohydrate diets apply. Managing these counterposing effects might include reducing the intake of proteins with high concentrations of sulfur- and branched chain amino acids linked to pro-aging and disease pathways (Hine et al., 2015; Solon-Biet et al., 2014), decreasing dietary P:C by replacing dietary fats with healthy carbohydrates, periods of intermittent fasting, and drug development targeting nutrient-sensing pathways (Le Couteur et al., 2012; Baur et al., 2012; Mattson et al., 2014).

Age itself is a major determinant of what constitutes an optimal diet. Hence, whereas low P:C diets benefit late life health and longevity (Levine et al., 2014), they are not optimal for somatic growth and reproduction earlier in life, which require higher protein intakes. In addition to age, a network of interacting factors need to be considered to determine an optimal diet, including genotype, epigenotype, sex, health, and immune status, commensal ecology, societal context, physical environment, and the level of activity.

Nutritional Geometry at the Cellular and Molecular Level

Mapping response landscapes as a function of multiple nutrient dimensions offers a step-change in understanding the nutritional phenotype of an animal, compared to energy or single-nutrient-based single-dimensional approaches. The same potential applies to deciphering cellular and molecular pathways. The concept that appetite and metabolism respond to specific nutrients and nutrient ratios is transformative for dissecting cellular mechanisms for these processes, evidenced by the recent discovery of FGF 21 as the first known candidate endocrine signal in the control of protein intake (e.g., Laeger et al., 2014). A geometric analysis can also better aid interpretation of the effects of genetic or pharmacological manipulations (Piper et al., 2011).

As an example of the use of nutritional geometry, a number of interacting nutrient-sensing pathways are considered to mediate the link between diet and aging, including mTOR, AMPK, insulin/IGF1/GH, and SIRT1. The effects of dietary P:C on lifespan in mice and flies led to the prediction that these pathways, either individually or in combination, are responsive to P:C ratio rather than to energy or single nutrients (Simpson and Raubenheimer, 2009). This hypothesis was supported by response surface analyses indicating that circulating insulin levels were strongly influenced by dietary P:C, and that hepatic mTOR activation was a positive function of the ratio of circulating branched chain amino acids and glucose (Solon-Biet et al., 2014).

Food for Thought

Here we have focused on the relationships among diet composition, intake, and health, but nutritional geometry has also been used to investigate the broader causes of variance in diet composition of humans and other animals, including developmental, economic, evolutionary, and ecological (Raubenheimer et al., 2015). This intake-focused approach is not an alternative to theories of human nutrition that center on variation in biological responses to ingested nutrients, for example the propensity to store fat (Wells, 2006). Rather, as stressed by Speakman (2014), nutrient intake and its consequences are best modeled as part of the same system, enabling the understanding, prediction, and management of organism- and population-level responses to different environments (Lihoreau et al., 2014). We stress, further, that nutrient combinations entered into a geometric model should be considered on a case-by-case basis. To date many questions have been addressed by modeling interactions among the macronutrients (Simpson and Raubenheimer 2012), but in other cases mineral micronutrients and

vitamins (e.g., Blumfield et al., 2012) or specific amino acids (Solon-Biet et al., 2014) have been integrated into the model. The quality of macronutrients (types of fats, carbohydrates, and proteins) is another important aspect of diet that is amenable to geometric analysis, yet remains uncharted. It is only through acknowledging the complexity of nutrition and systematically charting its implications from the food environment to dietary choices and health consequences that we can hope to tame the epidemic of obesity-related diseases that has arisen over recent decades.

ACKNOWLEDGMENTS

We acknowledge funding support from the National Health and Medical Research Council, Australian Research Council, Gravida (The National Research Centre for Growth and Development, New Zealand), and the Ageing and Alzheimers Institute for supporting the fruit fly and mice studies discussed in this essay. We also thank our colleagues who contributed to these studies.

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Note Added in Proof

Since the submission of this manuscript, an additional paper has shown that reproductive function is best supported in male and female mice on a higher-protein diet.

Solon-Biet, S.M., Walters, K.A., Simanainen, U., McMahon, A.C., Ruohonen, K., Ballard, J.W.O., Raubenheimer, D., Handelsman, D.J., Le Couteur, D.G., and Simpson, S.J. (2015). Macronutrient balance, reproductive function and lifespan in aging mice. Proceedings of the National Academy of Science, USA *112*, 3481–3486.

Cell

Leading Edge
Perspective

Multisensory Flavor Perception

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http://dx.doi.org/10.1016/j.cell.2015.03.007

The perception of flavor is perhaps the most multisensory of our everyday experiences. The latest research by psychologists and cognitive neuroscientists increasingly reveals the complex multisensory interactions that give rise to the flavor experiences we all know and love, demonstrating how they rely on the integration of cues from all of the human senses. This Perspective explores the contributions of distinct senses to our perception of food and the growing realization that the same rules of multisensory integration that have been thoroughly explored in interactions between audition, vision, and touch may also explain the combination of the (admittedly harder to study) flavor senses. Academic advances are now spilling out into the real world, with chefs and food industry increasingly taking the latest scientific findings on board in their food design.

Introduction

According to many authors, foraging and feeding are among the most important of the everyday tasks that our brains have evolved to deal with. As J.Z. Young (1968, p. 21), the eminent British biologist, once put it, "No animal can live without food. Let us then pursue the corollary of this: Namely, food is about the most important influence in determining the organization of the brain and the behavior that the brain organization dictates."

Indeed, some of the most dramatic changes in brain activity are seen when a hungry participant is presented with appetizing food images while lying passively in the brain scanner (van der Laan et al., 2011). It can therefore be argued that, even if one is not interested in flavor perception per se, ultimately studying the perception of food and drink may be central to our understanding of brain function.

However, despite its obvious importance, psychologists and cognitive neuroscientists have been slow to show much interest in studying flavor perception. In part, this neglect may reflect the difficulty of controlling stimulus delivery (this kind of research can't be done with a participant sitting obediently in front of a PC). Part of the problem, I think, also links to the fact that subjects rapidly adapt and hence may become sated after a few presentations of the experimental stimuli. This often necessitates multiple testing sessions. However, neglect of this field may also link to a more deep-seated belief that taste and smell constitute "lower," or "common," senses. Such a view is captured by the following quote from William James from a little over a century ago: "Taste, smell, as well as hunger, thirst, nausea and other so-called 'common' sensations need not be touched on…as almost nothing of psychological interest is known concerning them." One sometimes finds oneself wondering just how much has changed in the intervening years!

One of the most intriguing facts about the sense of taste is that we are all, in a very real sense, born into different taste worlds. Indeed, individual differences in taste receptor density on the tongue are far higher than for any of the other senses. To give you an idea, some people (called supertasters) have 16 times more taste buds on their tongues than other individuals—the non-tasters (see Bartoshuk, 2000). That said, the latest research suggests that the profound differences in people's sensitivity to bitter-tasting foods, such as cruciferous vegetables like Brussels sprouts and lab compounds such as propylthiouracil PROP, depend far more on the status of the PROP receptor encoded by the TAS2R38 gene than on the density of taste buds (Garneau et al., 2014). Supertasters are also more sensitive to the oral-somatosensory attributes of foods, such as the fat in a salad dressing (Eldeghaidy et al., 2011). Expertise, as for instance in wine tasters, has also been shown to predict taste phenotype (Hayes and Pickering, 2012).

Flavor involves the combination of gustatory and olfactory stimuli, giving rise to descriptors such as "fruity," "meaty," "floral," "herbal," etc. Here, it is important to distinguish between orthonasal smell when we sniff (that tells us about the aroma of food, the bouquet of the wine) and the retronasal smell when air is pulsed out from the back of the nose as we swallow (e.g., Rozin, 1982). While the distinction between these two senses of smell has been recognized for more than a century (see Shepherd, 2012), only recently have researchers been able to provide empirical support for the claim that different neural substrates may actually be involved in processing these two kinds of olfactory information (see Small et al., 2005). It is the retronasal aromas that are combined with gustatory cues to give rise to flavors. On top of these two senses, trigeminal inputs also contribute to flavor perception. As for the other senses, such as vision, audition, and oral somatosensation, the jury is currently still out as to which if any of these senses should be considered as constitutive of flavor perception or, rather, as factors that merely modulate the experience of flavor (see Spence and Piqueras-Fiszman, 2014).

Olfactory-Gustatory Interactions underlying Multisensory Flavor Perception

While it is only natural to think of taste (i.e., gustation) as playing a key role in multisensory flavor perception, the majority of

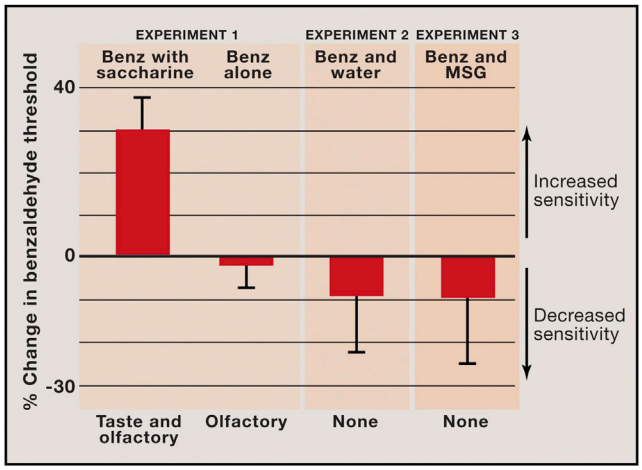

Figure 1. Multisensory Interactions between Olfaction and Gustation in Multisensory Flavor Perception

Results of a series of experiments by Dalton et al. (2000), showing the integration of orthonasal olfactory and gustatory cues. Figure reprinted with permission from Figure 6.2 of Spence and Piqueras-Fiszman (2014).

commentators agree that it is the sense of smell (or olfaction) that actually contributes the majority of the information to our experience (see Spence et al., 2015). In fact, it has been suggested that as much as 80%–90% of the taste of food comes from the nose (e.g., Chartier, 2012; Stuckey, 2012), and we have all experienced food tasting of nothing much when we have a head cold, thus providing anecdotal support for the importance of olfactory input to the enjoyment of food and drink. That olfaction contributes disproportionately more to the experience of flavor seems an easy claim to accept (Murphy et al., 1977). That said, one might question whether it is possible to put a meaningful numerical value on this, given that the relative contribution of each of the senses presumably depends on the particular foodstuff under consideration—just compare your experience of a ripe French brie cheese to that of a water biscuit!

In the west, we describe the aromas of strawberry, caramel, and vanilla as smelling "sweet" (Stevenson and Boakes, 2004). (Those who have tried eating a raw vanilla pod know only too well how bitter it actually tastes.) It turns out that this is more than merely a synaesthetic or metaphorical use of language (Stevenson and Tomiczek, 2007). Olfactory stimuli that have regularly been paired with sweet, bitter, salty, or even sour-tasting foods can, in fact, come to enhance the associated taste quality, even when they are presented at a sub-threshold level. There can be no doubt that such crossmodal interactions make it all the more difficult to try and draw a clear line between experiences of taste and of flavor (see Spence et al., 2015). No wonder then that philosophers, too, are starting to take an interest in some of the thornier problems raised by the study of flavor perception. Stevenson (2009, pp. 3–4) succinctly captures one of the central issues for the philosopher when he notes that, "It is possible to conceive of flavor in several ways; as a multimodal object, a sensory system, a unique sense in and of itself, and a set of discrete senses bound together by centrally mediated processes…Flavor is clearly multimodal, but where does one draw the boundary? After all, visual and auditory stimuli influence flavor perception, so are they part of a flavor sense? One way of navigating around these issues is to regard all of the senses that contribute to flavor, as part of a flavor system (as so far done…), but to retain the term 'flavor' for the stimulus experienced in the mouth."

Some of the most convincing evidence concerning the multisensory integration of orthonasal olfactory and gustatory cues comes from seminal research conducted by Pam Dalton and her colleagues at the Monell Chemical Senses Center in Philadelphia (Dalton et al., 2000). Participants in their studies were given two pairs of bottles to sniff, each containing a clear odorless liquid. An almond-cherry-like scent (i.e., benzaldehyde) had been added to one of the bottles. On each trial, the participants had to try and determine which bottle contained the benzaldehyde. The concentration of the olfactant was varied on a trial-by-trial basis in order to home in on each participant's detection threshold. Surprisingly, when the participants performed this task while holding a sub-threshold solution of saccharin in their mouths (i.e., a solution that had no discernible taste or smell), the cherry-almond smell was perceived as being significantly more intense relative to a baseline condition in which a tasteless water solution was held in the mouth instead (see Figure 1). By contrast, holding a sub-threshold solution of monosodium glutamate (MSG) on the tongue did not give rise to any such change in the ability of Dalton et al.'s participants to smell the aroma in the bottle. Taken together, such a pattern of results highlights the stimulus-specific integration of tastants and olfactory stimuli (a specificity that turns out to be characteristic of a number of the studies that have been published in this area; see Spence, 2012 for a review).

Similar results have now been reported in several subsequent studies. For instance, Pfeiffer et al. (2005) demonstrated a 50% lowering of the olfactory threshold—that is, complete additivity in the majority of their participants when the relevant gustatory and olfactory stimuli were presented simultaneously. Intriguingly, similar results were observed regardless of whether the odor was delivered orthonasally or retronasally. And moving the experimental situation even closer to everyday life, similar effects have now been reported with participants tasting actual flavored solutions (see Delwiche and Heffelfinger, 2005).

There is also an intriguing cross-cultural angle to this research. Japanese participants tend to show perceptual enhancement in the MSG condition, but not in the saccharin condition (i.e., the opposite pattern to that shown by western participants in Dalton et al., 2000; see Breslin et al. 2001). It turns out that pickled condiments containing the savory almond combination are common in Japanese cuisine, whereas sweet almond desserts (just think of Bakewell Tart) are more commonly experienced in the west. These results therefore suggest that our brains learn to combine tastes and smells that regularly co-occur in our home cuisine. The underlying idea here then is that, while everyone's brain may use the same rules to combine the inputs from their senses, the particular combinations of tastants and olfactory stimuli (and possibly also visual stimuli) that lead to multisensory enhancement (or suppression, when the taste and smell don't match; see, e.g., de Araujo et al., 2003) depends on the combination of ingredients and, hence, of sensory cues that tend to co-occur

in the cuisine of the region where people have grown up. Such learning apparently starts in utero (see Schaal et al., 2000; see Bremner et al., 2012 for a review). French researchers have, for example, demonstrated that neonates whose mothers consumed anise-flavored food during pregnancy are more likely to orient toward the smell of anise after birth, while elsewhere it has been shown that young children are more likely to eat carrots if their mothers happened to drink carrot-flavored milk during pregnancy.

Visual Contributions to Multisensory Flavor Perception

Moir (1936), a chemist by training, was perhaps the first to report that simply changing the color of food could affect people's perception of taste/flavor. In the years since, more than 150 further studies examining vision's influence over taste and flavor have been published. The majority, but by no means all, of this research has demonstrated that changing the hue and/or intensity of the color added to a food or, more frequently, a beverage can influence the perceived identity and/or intensity of the flavor. While varying the color intensity impacts the rated taste and flavor intensity in some studies, such a crossmodal effect is not always found (see Spence et al., 2010 for a review). The reasons behind such mixed results may well be explained by the different taste/flavor expectations that can sometimes be associated by different people with one and the same food color (see below).

One of the most common observations has been that changing the hue of a drink changes the perceived flavor. Many people will, for example, say that a cherry-flavored drink tastes of lime if colored green while perceiving it to taste of orange if colored orange (see Zampini et al., 2007, 2008). One intriguing but as yet unconfirmed observation in this area comes from Zampini et al. (2008). These researchers found that supertasters were significantly less influenced by inappropriate coloring of a beverage than were medium tasters who, themselves, were less influenced than were the non-tasters (see Figure 2).

In one of the classic studies, Morrot et al. (2001) investigated the effects of color on people's perception of wine aroma. These researchers were able to fool more than 50 students enrolled in a university wine degree course in Bordeaux, France into believing that they were holding a glass of red wine, simply by coloring a white Bordeaux wine artificially red with an odorless food dye! The participants were initially given a glass of white wine and instructed to describe its aroma. Next, they were given a glass of red wine and had to do the same. As one might have expected, the students used completely different terms in order to describe the aromas of the two wines—terms like citrus, lychee, straw, and lemon for the white wine and chocolate, berry, and tobacco to describe the red wine. Finally, the students were given a third glass of wine and had to decide which of the aroma terms that they had chosen previously constituted the best match for the wine. The third glass again looked like red wine but was, in fact, the white wine that the students had been originally given, colored so as to be visually indistinguishable from the red wine. Surprisingly, they mostly choose the red wine odor descriptors, apparently no longer perceiving the aromas that they had previously reported when drinking the untainted white wine. This result therefore powerfully demonstrates vision's dominance over orthonasal olfaction. Further, it is also consistent with other studies showing that "experts" are no less susceptible to such crossmodal effects than are regular consumers.

Similar results have subsequently been reported in New Zealand wine experts (including professional wine tasters and wine makers) who were actually allowed to taste the wine that they had to evaluate (Parr et al., 2003). In particular, the experts' descriptions of the aroma of a barrique-fermented young Chardonnay that had been artificially colored red were more accurate when the wine was served in an opaque glass than when it was served in a glass that was clear. Indeed, wine may well be the single most extensively studied drink when it comes to looking at the impact of color (and expertise) on the crossmodal influence of visual cues on multisensory flavor perception (see Spence, 2010, for a review).

Intriguingly, the crossmodal effects of color on both wine and fruit-flavored soft drinks occur even when participants are explicitly told to try and ignore what they see (Parr et al., 2003; Zampini et al., 2007, 2008). Such results hint at the automaticity of the crossmodal effects of vision (color) on flavor. It would appear that both the hue and intensity of the coloration automatically set expectations in the mind of the observer about the likely identity and intensity of the taste/flavor of food and drink. Remember that we nearly always see what we are going to eat or drink in advance (one of the few exceptions being the dine-in-the-dark restaurant, where, according to most published accounts, the food tastes disappointing; see Spence and Piqueras-Fiszman, 2014), and those expectations will either be confirmed or disconfirmed when a person comes to taste/evaluate the food or drink. Now, if the expected taste/flavor happens not to be too dissimilar from the actual taste/flavor, people will likely report that their experience matches their expectation. By contrast, should the discrepancy between what we expect and what we actually taste be too great, then a disconfirmation of expectation response is likely. One critical question here is how much of a discrepancy between the expectation and the experience is needed in order to trigger a disconfirmation of expectation response (see Shankar et al., 2010 on this topic).

It is important to remember that disconfirmed expectations can occur in both the sensory-discriminative and hedonic domains (Zellner et al., 2004; see Piqueras-Fiszman and Spence, 2015 for a review). In everyday life, such disconfirmation of expectation tends to be negatively evaluated, hence perhaps helping to explain the failure of clear cola drinks in the marketplace a few years back (e.g., see Triplett, 1994). That said, the influence of context cannot be ignored here. Indeed, it is intriguing to note how many people seem to positively relish the opportunity of having their expectations disconfirmed, providing that it occurs within the confines of the modernist restaurant (see Spence and Piqueras-Fiszman, 2014). It probably helps if they are neophilic rather than neophobic when it comes to experimenting with new foods. This pleasure in surprise is something that is difficult to capture (and hence study) in the setting of the laboratory. In fact, it may rely on our believing that we are in capable hands (e.g., of the star modernist chef), so interpreting the disconfirmation as a carefully crafted multisensory flavor experience rather than merely reflecting poor design.

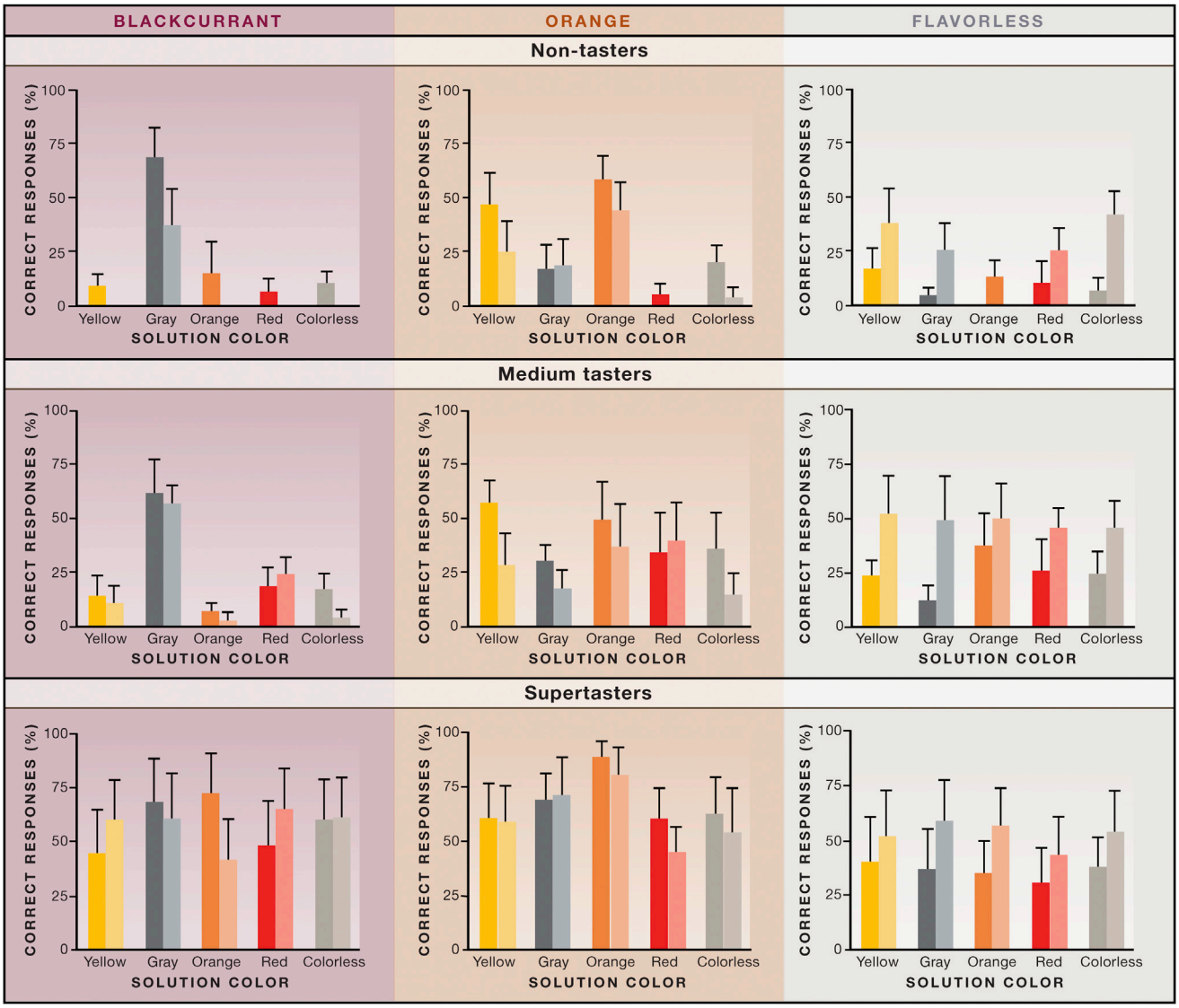

Figure 2. The Influence of Color on Flavor Identification as a Function of Taster Status

Mean percentages of correct flavor identification responses for the three groups of participants (non-tasters, medium tasters, and supertasters) for the blackcurrant, orange, and flavorless solutions presented in Zampini et al.'s study (2008) of the effects of color cues on multisensory flavor perception in humans. The darker columns represent solutions where fruit acids had been added, and the lighter columns represent solutions without fruit acids. The error bars represent the between-participants standard errors of the means. Figure reprinted with permission from Figure 1 of Zampini et al. (2007).

Children seem to enjoy artificially (i.e., brightly) colored and miscolored foods more than their parents (see Bremner et al., 2012). One example is the green-colored ketchup that was successfully launched into the marketplace a little over a decade ago or the miscolored candies, where the challenge for children is to try to ignore the evidence before their eyes and discern the product's actual flavor. At the opposite end of the age spectrum, it has been suggested that the intelligent use of food coloring might help to deliver better-tasting foods for those whose sense of taste and smell has started its inevitable decline. This impairment becomes especially noticeable when people reach their sixth or seventh decade (see Bremner et al., 2012), leading to unhealthy eating behaviors. For example, old people have to add as much as two to three times more salt to a bowl of tomato soup in order to achieve the same taste/flavor as young people (see Stevens et al., 1991). Worryingly, medications that are commonly used in these populations have been shown to increase the need for salt by as much as twelve times. Here one is reminded of the old line from Brillat-Savarin's 19th century classic text, *The Philosopher in the Kitchen*: "The pleasures of the table, belong to all times and all ages, to every country and to every day; they go hand in hand with all our other pleasures, outlast them, and remain to console us for their loss."

One aspect of the influence of color and other visual cues that has not received as much attention from researchers to date concerns the powerful aversive responses that off-colors in

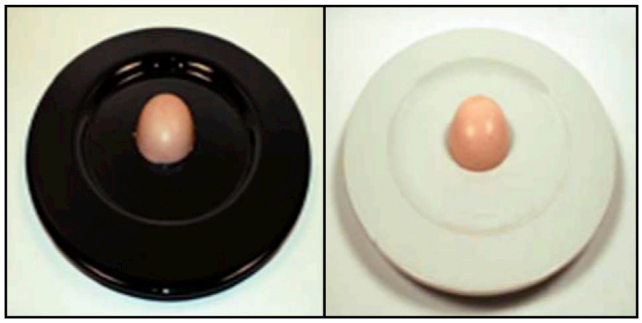

Figure 3. Can You Taste the Plate?
Black and white plates with red frozen strawberry dessert. Participants rated the dessert tasted from the white plate as tasting significantly sweeter and more flavorful than exactly the same food when served from the black plate instead. Figure reprinted with permission from Figure 2 of Piqueras-Fiszman et al. (2012).

foods can induce (see Spence and Piqueras-Fiszman, 2014 for a number of anecdotal examples). Early on, for example, Moir (1936) reported that a number of participants felt ill after his early experimentation on his work colleagues. As one commentator notes, "Moir prepared a buffet of foods for a dinner with scientific colleagues of the Flavor Group of the Society of Chemistry and Industry in London. Many of the foods were inappropriately colored, and during the dinner several individuals complained about the off-flavor of many of the foods served. Several of the individuals reported feeling ill after eating some of the foods, despite the fact that only the color was varied. The rest of the food was perfectly wholesome, with the requisite taste, smell and texture." There may, of course, have been a historical shift in the acceptability of unusual food coloring over the decades. Indeed, the indomitable Fanny Cradock, one of the UK's first celebrity chefs, may have a lot to answer for here as she was particularly fond of presenting foods such as mashed potatoes in a variety of psychedelic colors (though fortunately not all at once) in her television shows back in the 1960s and 70s.

Similarly, back in the 1970s, one mischievous marketer invited a group of friends over to dine on a meal of steak, chips, and peas (it was the 1970s, after all; Wheatley, 1973). The only thing that may have struck any of the guests as strange or unusual was how dim the lighting was. When the lighting was turned back up, imagine the guests' horror as they saw that they were actually eating a blue steak, green chips, and red peas. A number of them apparently started to feel decidedly ill, some heading for the bathroom. In a similar vein, Alfred Hitchcock used to enjoy discomforting his guests by serving entirely blue meals while entertaining at a private dining room at the Trocadero in London (see Spence and Piqueras-Fiszman, 2014, p. 224). The potential commercial relevance of such findings was highlighted by the negative response of people to a batch of brown but otherwise normal-tasting grapefruit juice donated to a food bank in 1981. As yet, it is unclear whether such aversive responses to such putatively off-colors in foods involve the same neural substrates as the other examples of visual dominance that have been described so far in this Perspective.

While the majority of research on visual contributions to flavor perception has focused on studying the impact of uniformly changing the color of a food or beverage item (see Spence et al., 2010 for a review), a growing body of research over the last 5 years has started to investigate how the background color of the plateware, glassware, and/or even the cutlery may influence taste and flavor perception. For example, in research conducted together with the Alicia-ElBulli Foundation in Spain, Piqueras-Fiszman et al. (2012) were able to demonstrate that exactly the same dessert, a frozen strawberry mousse, was rated as tasting 10% sweeter, 15% more flavorful, and significantly better liked when eaten from a white plate rather than a black plate (see Figure 3). Remarkably, follow-up research by Stewart and Goss (2013) has demonstrated even more striking effects of plateware on people's taste perception by varying both the color and shape of the plate on which a food was served. (It turns out that round plates are "sweeter" than more angular plates!)

Similarly, the perceived flavor of a hot chocolate drink has been shown to be influenced by the color of the cup in which it is served (Piqueras-Fiszman and Spence, 2012a). In particular, the chocolate flavor was rated as significantly more intense when the drink was served from an orange plastic cup than when served from a white cup. Indeed, there have been a number of anecdotal reports of customers complaining about the change in the taste of their favorite branded soft drinks, when the color of the beverage can was itself changed (e.g., see http://online.wsj.com/article/SB10001424052970204012004577070521211375302.html for one recent example).

Even the color of the cutlery can exert a small but significant influence on people's perception (or at least their rating) of the taste of yogurt (Harrar and Spence, 2013). While the explanation for results such as these has not yet been fully worked out, part of the answer may relate to color contrast. That is, the apparent color of the food may change subtly as a function of the background color against which it is seen (see Lyman, 1989). Part of the explanation may also relate to the prior associations that we have built up over the years between particular plateware/glassware and the taste and flavor experiences that have normally been associated with them (see also Wan et al., 2014a).

Beyond the color and shape of the plateware, an emerging body of research has started to look at the influence of the more complex arrangement of food on the plate (see Deroy et al., 2014; Spence et al., 2014a, 2014b for reviews). In one recent study, the young Franco-Colombian chef Charles Michel demonstrated that customers rated the same set of ingredients as tasting significantly better (and, what is more, they were willing to pay significantly more for the dish) when served in an arrangement inspired by one of Kandinsky's paintings than when served as a regular tossed salad or with the elements arranged carefully in a side-by-side manner instead (see Figure 4). While the original study was conducted in the laboratory setting, subsequent research has replicated the same finding in a restaurant setting (see Michel et al., 2015; Spence and Piqueras-Fiszman, 2014; see also Zellner et al., 2014).

I believe that, in the years to come, we are going to see a lot more research into how the more complex aspects of visual aesthetics, such as balance, harmony, and orientation, influence our

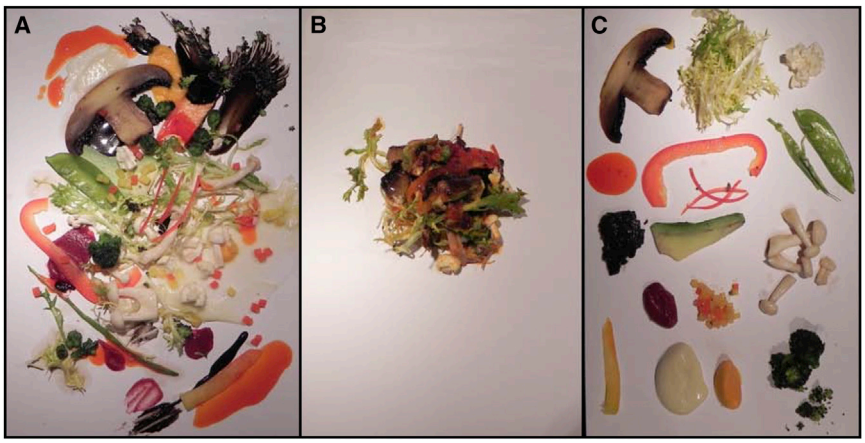

Figure 4. Salad Three Ways

(A) Three different presentations of exactly the same ingredients served to participants in Michel et al.'s Kandinsky on a plate study (2014a). Plating inspired by Kandinsky's "Painting number 201," hanging (the other way up) in the MoMA in New York (see http://www.moma.org/collection/browse_results.php?object_id=79452).

(B) Same ingredients now served as a regular tossed salad.

(C) The ingredients laid out side by side—an effortful presentation, but not an especially aesthetically pleasing one. No surprises for guessing that those participants served the artistic version of the salad liked it more and were willing to pay significantly more for the food. Figure adapted and reprinted with permission from Figure 1 of Michel et al. (2014a).

judgments of food on the plate (Michel et al., 2015; Zellner et al., 2014). Indeed, as Apicius, the Roman gourmand, is once purported to have said, "The first taste is always with the eyes." Certainly, a growing number of modernist chefs are starting to become interested in scientifically assessing the impact of plating on the appreciation of the food they serve. Conducting research over the Internet is also increasingly starting to allow both chefs and sensory scientists/psychologists to assess the impact of (sometimes subtle) changes in a dish's visual design on people's expectations, which as we have already seen, can play a surprisingly large part in determining responses of the diner to the actual dish (e.g., Michel et al., 2015; see also Wan et al., 2014a).

Indeed, the fact that visual presentation of food turns out to be especially important to our multisensory flavor experiences is consistent with the results of neuroimaging studies that have demonstrated increased activation in diverse brain regions when participants, especially hungry ones, view images of food (see van der Laan et al., 2011 for a review and meta-analysis). There is also an emerging literature looking at the attention-capturing potential of visual food images (e.g., see Harrar et al., 2011; Toepel et al., 2009). Once again, however, while food images turn out to be especially good at capturing our attention visually (we seem, in particular, to be drawn to those foods that have a high fat content), they have not, at least until very recently, been incorporated into the mainstream literature on spatial attention, for example.

Auditory Contributions to Multisensory Flavor Perception

It is almost 60 years since researchers first started thinking about the putative role of audition in the experience of food and drink (see Spence, 2015, for a review). Hearing always comes at the bottom of the list when people—whether they be professional sensory scientists or regular consumers—are asked to rank the relative importance of each of the senses to flavor perception (see Spence, 2015 on this point). Indeed, it was such neglect that led the famous British chef Heston Blumenthal (Blumenthal, 2008) to say of sound that it was "the forgotten flavor sense."

In the intervening years, a large body of sensory science research has been published, demonstrating that auditory cues do indeed play an important role in the multisensory perception of food attributes such as crispy, crackly, crunchy, carbonated, and even creamy (see Spence, 2015). And while a number of these might initially seem to be oral-somatosensory in nature, it turns out that our in-mouth experience can be radically changed by modifying the sounds of mastication. So, for example, in one of the classic studies (which was awarded the 2008 IG Nobel prize for nutrition), Zampini and Spence (2004) demonstrated that people's perception of the crispness and freshness of potato chips (crisps to the readers in the UK) could be systematically modified (by around 15%) by changing the self-generated crisp biting sounds that participants heard when biting into a selection of this not altogether healthy dry snack food (see Figure 5).

Recently, the very same approach has been extended to demonstrate the role of auditory cues in the perception of the crispness of a moist crisp food, namely apples (see Dematte et al., 2014). What is perhaps worth noting here is that the "sonic chip" paradigm came not from the sensory science labs but was, in fact, adapted from a psychophysical test that had been developed originally to measure the parchment skin illusion. In this crossmodal illusion, participants are encouraged to rub their hands together while listening to the rubbing sounds picked up by a microphone and played back in real time over headphones. Simply by changing the sound that people hear, either boosting or cutting certain components of the frequency spectrum, the person's hands switch from feeling dry and patched one moment to moist and clammy the next (Guest et al., 2002; Jousmäki and Hari, 1998). This is an example of how insights from the study of audition, touch, and vision can provide insights and approaches that are useful when it comes to trying to understand the multisensory nature of human flavor perception.

Oral-Somatosensory Contributions to Multisensory Flavor Perception

Assessing the contribution of oral-somatosensory cues to multisensory flavor perception is undeniably hard. As such, we have not as yet learned as much about the undoubtedly important role of this sense in delivering the creamy, oily, velvety crispy, crunchy, etc. attributes of food and drink as we might like

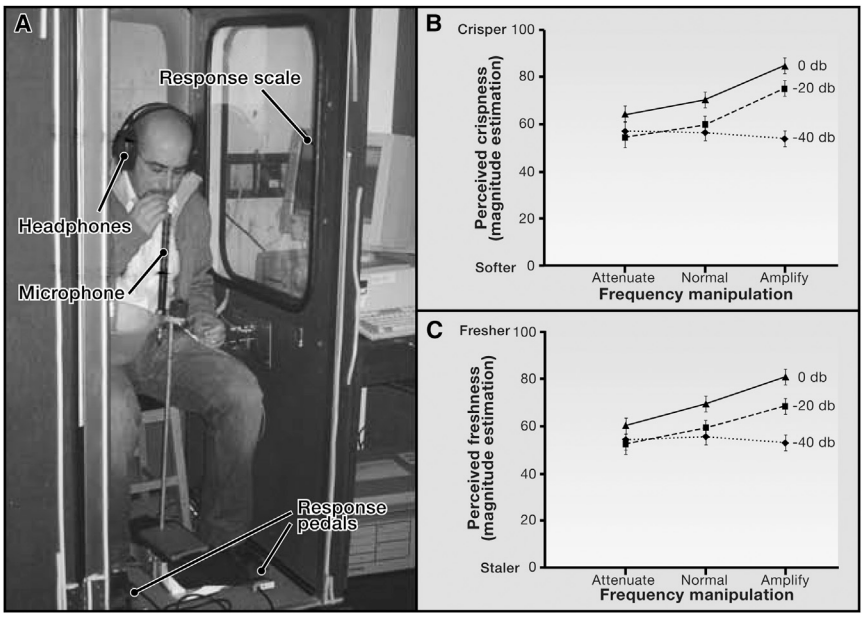

Figure 5. The "Sonic Chip" Experiment

(A) Schematic view of the apparatus and participant in Zampini and Spence (2004) study demonstrating the influence of biting sounds on crispness and freshness perception. The door of the experimental booth was closed during the experiment, and the response scale was viewed through the window in the left-side wall of the booth. Mean responses for the soft-crisp (B), and fresh-stale (C) response scales for the three overall attenuation levels (0 dB, −20 dB, or −40 dB) against the three frequency manipulations (high frequencies attenuated, veridical auditory feedback, or high frequencies amplified) are reported. Error bars represent the between-participants standard errors of the means. Figure reprinted with permission from Figure 1 of Zampini and Spence (2004).

(though see Bult et al., 2007; De Araujo and Rolls, 2004; Eldeghaidy et al., 2011 for some of the best evidence published to date). In one intriguing study, Bult and his colleagues delivered a creamy odor either orthonasally or retronasally (via an olfactometer). At the same time, milk-like substances with different viscosities were delivered to the participant's mouth. The latter was instructed to rate the intensity of the flavor, as well as the creaminess and thickness of the resulting in-mouth experience. Ratings of flavor intensity decreased as the viscosity of the liquid increased. This was true regardless of whether the olfactory stimulus was delivered via the orthonasal or retronasal route. These results highlight the important and complex role that texture (including mouthfeel) can play in the multisensory perception of flavor. Intriguingly, it turns out that retronasally delivered odors can also influence the perceived thickness of substances in the mouth (see also Roudnitzky et al., 2011).

Oral-somatosensory cues may also play an important role in localizing the flavor of food and drink to our mouths (Todrank and Bartoshuk, 1991; though see also Stevenson, 2014). Note that, while little studied to date, attention may play a role here too (see Stevenson, 2012 for a review). Researchers have speculated on the apparent similarities between "oral referral" and the ventriloquism effect that we all experience whenever we mislocalize the sounds of the actors' voices to the lips we see moving on the big screen at the cinema. Once again, given the challenges associated with studying oral referral directly, the hope is that our extensive understanding of the mechanisms of sensory dominance underlying the ventriloquism effect can be helpful to further our understanding of why what we smell retronasally gets mislocalized into the oral cavity, hence giving rise to what we commonly call the taste of food and drink.

One intriguing area of this research relates to the question of whether out-of-the-body illusions (such as "the rubber hand illusion," a kind of visual dominance over touch and proprioception; see the relevant chapters in Bremner et al., 2012; Stein, 2012 for reviews) can be extended to the tongue. Last year, Michel et al. (2014b) elicited the illusion, named the "butcher's tongue," by situating a rubber tongue in a plausible position just in front of the participant's own mouth and stroking one of the two tongues with cotton swabs, leading the participants to feel the touch on their own tongue by seeing the rubber tongue being stroked. The butcher's tongue illusion potentially offers one route to taking flavors out of the mouth in the future. Beyond the feel of the food, even the weight of the cutlery or bowl in the hand have been shown to influence taste perception and expected satiety were the food to be consumed (e.g., Piqueras-Fiszman and Spence, 2012b; Spence and Piqueras-Fiszman, 2014). The feeling of food in the hand has also been shown to modulate the oral perception of texture (see Barnett-Cowan, 2010). It turns out that the tactile feel of a pretzel while held in hand influences the oral perception of the pretzel. The softer the pretzel in the hand, the staler it was perceived in the mouth, thus suggesting something of a contrast effect. Here, one is reminded of the Italian Futurists, such as most famously Filippo Tommaso Marinetti (1876–1944), and their tactile dinner parties. In order to maximally stimulate the sense of touch, diners were encouraged to wear pajamas made of differently textured materials such as cork, sponge, sandpaper, and/or felt and to eat without the aid of knives and forks (see Spence and Piqueras-Fiszman, 2014, p. 291). In summary, touch, no matter whether in the mouth or on the hand, has a far greater influence on perception of taste, quality, and satiety than any of us realize.

Ambient/Atmospheric Cues and Their Influence on Multisensory Flavor Perception

Beyond intrinsic cues of the food and their influence on the multisensory perception of flavor, it is important to note that ambient lighting, background music, and background noise have all been shown to influence taste and flavor perception (e.g., see Oberfeld et al., 2009; Spence, 2014; Spence et al., 2014a, 2014b; Velasco et al., 2013). For instance, in one experiment, almost 3,000 people were given a red wine to taste from a black tasting glass (so that they could not discern the

drink's actual color). The wine was liked significantly more, and it was rated as tasting significantly fruitier when the participants found themselves in an environment with red ambient lighting and putatively sweet background music than when the lighting was turned to green and sour music was played instead (see Knöferle et al., 2015 on the development of music to match each of the four basic tastes). A growing body of research now shows that, when asked to match tastes and flavors with a particular pitch of sound or with a specific class of musical instrument, their responses are non-random (see Knöferle & Spence, 2012 for a review). The majority of people will, for example, match sweet-tasting foods with sounds having a higher pitch and the sound of the piano while matching bitter-tasting foods with lower pitched sounds and the sound of a brass instrument.

There is also much research showing that the atmosphere in the restaurant can influence taste/flavor perception, as well as the perceived ethnicity of a dish (see Spence and Piqueras-Fiszman, 2014 for a review). It is an open question though as to whether such crossmodal effects are best conceptualized in terms of sensation transference or some kind of perceptual/semantic priming effect. Nevertheless, whatever the correct explanation, it is no surprise to see that such results are of growing interest to the restaurateurs and food marketers.

Cognitive Influences on Multisensory Flavor Perception

While the majority of this Perspective has focused on the role of multisensory interactions in flavor perception (that has most typically been studied in the laboratory), in the real world, cognitive factors such as branding, labeling, packaging, and pricing also play an important role in determining our sensory-discriminative and hedonic expectations (see Piqueras-Fiszman and Spence, 2015 for a review). Certainly, there is good evidence to suggest that our cognitive expectations regarding taste or flavor can have a profound influence on some of the earliest neural sites where olfactory and gustatory information are first processed (see Grabenhorst et al., 2008; McClure et al., 2004; Plassmann et al., 2008). Even reading the word salt, for example, has been shown to activate many of the same areas as when a salty taste is actually experienced in mouth (see Barrós-Loscertales et al., 2012).

The description of a food plays a particularly important role when the expectations upon seeing a dish (what is sometimes referred to as the "visual flavor") are either ambiguous or different from the actual taste or flavor of the dish. This idea was beautifully demonstrated in a now-classic study by Martin Yeomans and his colleagues (including Heston Blumenthal) at the University of Sussex (Yeomans et al., 2008). In this collaboration, three groups of participants were given a pinkish-red ice-cream to taste. One group was given no information about the dish. A second group was informed that they would be tasting a savory ice-cream. Meanwhile, a third group of participants was told that they would be tasting a novel food called "Food 398." Those participants who hadn't been given any information about the smoked-salmon-flavored food (and who presumably would have been expecting to taste a sweet berry-flavored ice-cream based on the information before their eyes) rated the dish as tasting much saltier than either of the other two groups. What is more, these participants reported liking the dish far less, presumably due to the occurrence of a strong disconfirmation of expectation response.

Results such as these nicely illustrate how the meaning of what consumers see (or, in other words, their expectation concerning a food or drink's likely flavor and how much they would enjoy it) can be radically changed as a function of additional information that they may have been given about the food. Note that such higher-order effects of labeling tend to be especially pronounced when the stimulus itself is in some sense ambiguous. Indeed, several studies have now shown that ambiguous odors such as, for example, isovaleric acid, will light up different parts of the brain (specifically the orbitofrontal cortex) as a function of whether the smell has been described to the participant as a ripe cheese or as the distinctive smell of sweaty socks.

Perhaps understandably, given such results, some of those working a little closer to the marketing end of food research have criticized much of the laboratory research that has been published to date for focusing so much on the purely perceptual interactions taking place in flavor perception. The suggestion is that such research is in danger of underplaying the role of all the other higher-level cognitive factors that typically influence (or constrain) our expectations and hence our experience of food consumption.

Crossmodal Correspondences and Flavor Perception

One growing area of interest in the study of multisensory flavor perception comes from work on crossmodal correspondences. The latest research shows that people tend to associate tastes, food aromas, and flavors with other unrelated sensory cues in ways that are surprisingly consistent. For example, as we saw earlier, people tend to match sweet tastes with high-pitched sounds and the sound of the piano, while matching bitter tastes with low-pitched brassy sounds instead (see Knöferle and Spence, 2012 for a review). However, people also reliably match sweetness with roundness and redness. The other tastes (bitter, salty, sour, and umami) are also typically matched with particular colors and, if anything, with shapes that are more angular (see Velasco et al., 2015). A growing body of research is now coming to document the range of crossmodal correspondences in the world of taste, aroma, and flavor. While the origin of many of these correspondences is still being debated, it is exciting to see a growing number of young chefs who are starting to incorporate these findings in the design of their dishes (e.g., see Figure 6).

Neural Circuits underlying Multisensory Flavor Perception

The last few years have seen a rapid growth in our understanding of the neural networks that underlie multisensory flavor perception (see Shepherd 2012; Small, 2012 for reviews). Gustatory stimuli project from the tongue to the primary taste cortex (more specifically, the anterior insula and the frontal or parietal operculum), whereas olfactory stimuli project directly to the primary olfactory (i.e., piriform) cortex. From there, the inputs from both senses project to the orbitofrontal cortex (OFC). Gustatory stimuli are thought to project to caudolateral OFC, whereas

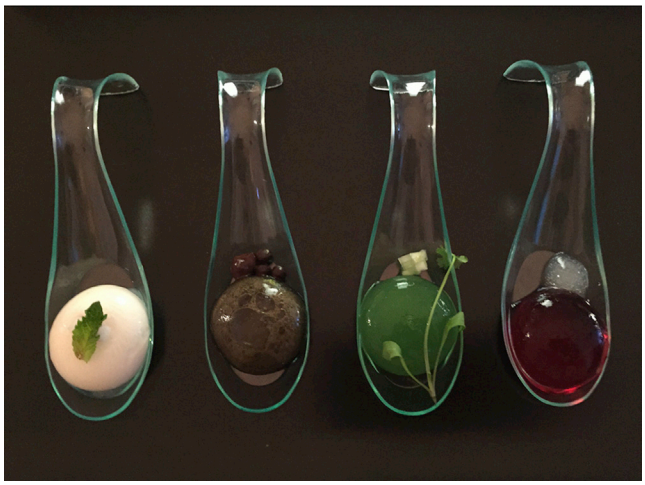

Figure 6. Salty, Bitter, Sour, and Sweet
The four amuse bouche served at Synaesthesia by *Kitchen Theory* (see https://kitchen-theory.com/). The spoons are brought to the table in a random arrangement, and it is the diner's job to sort the tastes by color. The spoons in the figure are shown in the intended order. This dish was inspired by the latest cross-cultural research demonstrating the robust crossmodal correspondences that exist between color and taste (see Wan et al., 2014b). Picture adapted and reprinted with permission from Eva-Luise Schwarz/FOUR magazine.

olfactory stimuli project to caudomedial OFC. The OFC plays a central role in representing the pleasantness (and reward value) of a food or drink (Small, 2012). The participants in one influential neuroimaging study of multisensory flavor perception had to lie still in a scanner while rating the pleasantness and congruency of various different pairings of orthonasal olfactory and gustatory stimuli (de Araujo et al., 2003). The olfactory stimuli consisted of methianol (which smells like chicken broth) and strawberry odor. The tastants were delivered in a solution and consisted of sucrose and MSG. The participants received both congruent (e.g., strawberry odor and sucrose) and incongruent (e.g., chicken broth odor and sucrose) combinations of orthonasal olfactory and gustatory stimuli. Increased OFC activity was correlated with increased ratings of the pleasantness and congruency of the olfactory-gustatory stimulus pairing that the participants were evaluating. Thus, it would appear as though the presentation of familiar (or congruent) combinations of olfactory (both orthonasal and retronasal) and gustatory stimuli can lead to enhanced neural responses in parts of the brain that code for the hedonic (i.e., pleasantness) and reward value of food. Similar results have also been reported following the presentation of congruent combinations of visual and olfactory stimuli as well—think only of the smell of strawberries and the color red (Österbauer et al., 2005).

Dana Small and her colleagues presented familiar/unfamiliar combinations of retronasal olfactory and gustatory stimuli to participants (Small et al. 2004). Superadditive neural interactions (see Stein, 2012) were observed in the OFC for familiar (or congruent, sweet-vanilla), but not for unfamiliar (or incongruent) combinations of stimuli (such as for the salty-vanilla stimulus combination). Several other areas—including the dorsal insula, the frontal operculum, and the anterior cingulate cortex—also lit up, thus constituting what could perhaps be thought of as a "flavor network" (e.g., Shepherd 2012; Small 2012).

Conclusions

As this Perspective has hopefully made clear, there has been a rapid and long overdue growth of interest in the study of multisensory flavor perception in recent years. While those researchers interested in the topic have long recognized the key role played by gustatory, olfactory, and to a lesser extent trigeminal inputs, the last decade or so has seen an explosion of new research demonstrating the impact of visual, auditory, and oral-somatosensory cues in modulating our experience of food and drink. Part of the excitement undoubtedly stems from the growing realization that many of the same neural principles known to constrain the integration of the spatial senses of vision, audition, and touch (see Bremner et al., 2012; Calvert et al., 2004; Stein, 2012 for reviews) might also help to explain the integration of sensory cues giving rise to the multisensory flavor perception.

When thinking about multisensory flavor perception, it is important to distinguish between flavor expectations and flavor experiences (Stevenson, 2009). Under the majority of everyday conditions, the former have a profound influence on the latter. However, one of the questions without a clear answer is whether the same processes of multisensory integration are involved in both cases (acknowledging, of course, the differing combination of senses involved; Stevenson, 2009). Indeed, there is intriguing preliminary evidence, at both the behavioral and neural levels, to suggest that there may be some important differences (e.g., Koza et al., 2005; Small et al., 2005, 2008; Zampini and Spence, 2005).

It would seem likely that in the years to come there will be growing interest in the developmental study of multisensory flavor perception (across the whole lifespan; see Bremner et al., 2012) in the role of culture and prior experience in determining which combinations of flavor cues (e.g., gustatory, olfactory, and visual stimuli) give rise to multisensory integration (Blumenthal, 2008; Breslin et al., 2001; Wan et al., 2014b). Studying the role of individual and genetic differences (e.g., in taster status) and their role in modulating multisensory flavor perception will likely also become increasingly popular (Bartoshuk, 2000; Eldeghaidy et al., 2011; Zampini et al., 2008). Our growing understanding in this area will likely also be aided by a better understanding of the neural circuits involved in flavor perception (see Shepherd, 2012; Small, 2012 for reviews) and the as-yet understudied role of attention in processing and, more importantly, binding of flavor cues (see Stevenson, 2012).

Let me end by highlighting the growing optimism that some of the latest insights (e.g., increasing perceived sweetness by changing the color of food or by changing the color of the plateware on which it is served) can be utilized to help nudge consumers toward healthier food behaviors (Spence and Piqueras-Fiszman, 2014; see also Marteau et al., 2012). Given the commercial opportunities associated with a better cognitive neuroscience understanding of the multisensory nature of flavor perception and the crucial individual, developmental, and cultural differences therein, it should come as little surprise that

many of the world's largest food/flavor companies (think Nestlé, Unilever, and P&G on the one hand and Givaudan, Firmenich, and IFF on the other) have been investing heavily (not to mention publishing more than occasionally) in this area. That said, longer-term follow-up studies are still needed to really know how long-lasting some of these crossmodal effects are likely to be (cf., De Graaf et al., 1997), keeping in mind the fact that any multisensory interactions in flavor perception need to be considered within the wider context of branding, labeling, packaging, pricing, and other more cognitive factors that both constrain and influence our everyday experience of food and drink.

ACKNOWLEDGMENTS

The author would like to acknowledge the AHRC Rethinking the Senses grant (AH/L007053/1).

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Cultivating Healthy Growth and Nutrition through the Gut Microbiota

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http://dx.doi.org/10.1016/j.cell.2015.03.013

Microbiota assembly is perturbed in children with undernutrition, resulting in persistent microbiota immaturity that is not rescued by current nutritional interventions. Evidence is accumulating that this immaturity is causally related to the pathogenesis of undernutrition and its lingering sequelae. Preclinical models in which human gut communities are replicated in gnotobiotic mice have provided an opportunity to identify and predict the effects of different dietary ingredients on microbiota structure, expressed functions, and host biology. This capacity sets the stage for proof-of-concept tests designed to deliberately shape the developmental trajectory and configurations of microbiota in children representing different geographies, cultural traditions, and states of health. Developing these capabilities for microbial stewardship is timely given the global health burden of childhood undernutrition, the effects of changing eating practices brought about by globalization, and the realization that affordable nutritious foods need to be developed to enhance our capacity to cultivate healthier microbiota in populations at risk for poor nutrition.

Introduction

Understanding the determinants of the nutritional value of different foods has never been more important, with population stabilization being unlikely this century (Gerland et al., 2014) and growing challenges related to sustainable agriculture. An integral part of understanding how best to deliver nutritious food to a burgeoning population is understanding how the microbial community in our gut (the microbiota) is shaped by what we eat and how that community in turn shapes our development and health. Nowhere will this kind of insight be more crucial than in raising the world's children.

Current obstacles to achieving healthy and productive lives and societies are reflected in the United Nations' millennium development goals that include reductions in child mortality and hunger and improvements in maternal health (http://www.un.org/millenniumgoals/). The scope of the problem of childhood undernutrition is described by parameters such as the International Food Policy Research Institute's Global Hunger Index (http://www.ifpri.org/publication/2014-global-hunger-index), which is an aggregate measure of calorie intake plus the rates of children being underweight and childhood mortality within a given region or country.

Much has been said about how changing patterns of food preferences brought about by economic development, globalization, and changes in food technology and food distribution systems are producing dramatic changes in how, what, and when we eat. These changes, combined with rapid population expansion and issues related to sustainable agriculture, create the need and the opportunity to drive innovation in the area of identifying new, affordable, and nutritious foods. Here, we focus on the importance of understanding the postnatal developmental biology of our gut microbial community—a highly adaptable microbial "organ" that is critically involved in the biotransformation of foods to products that can shape many aspects of human biology. In our view, studies of human gut microbial communities will markedly revise current thinking about many aspects of human nutrition. The knowledge gained could and should catalyze efforts to integrate agricultural policies, food production, and nutritional recommendations for consumers representing different ages, cultural traditions, and geographies. Preclinical research platforms are now available to evaluate the effects of foods that we currently consume and those that we envision creating in the future on the gut microbial community and host biology in ways that can inform clinical studies. Furthermore, studies of children with undernutrition are highlighting the importance of postnatal development of the gut microbiota for achieving healthy growth and providing us with a new set of metrics to define the efficacy of nutritional recommendations and interventions directed at infants, the maternal-infant dyad, and children. Finally, we emphasize the importance of addressing ethical, social, and regulatory issues related to research in this area now rather than later.

Defining Human Postnatal Development from a Microbial Perspective

The human gut microbiota is composed of all three domains of life; Bacteria, which predominate, Archaea, and Eukarya, plus

viruses. The gut microbiota is composed of relatively few bacterial phyla compared to communities in other body habitats and is notable for its strain-level diversity. Application of low-error sequencing methods to PCR amplicons generated from the bacterial phylogenetic marker gene encoding the principal RNA in the small subunit of ribosomes (16S rRNA) has indicated that, once acquired, the majority of bacterial strains in a healthy adult are retained for long periods of time (Faith et al., 2013). Thus, early colonizers, once established in the gut ecosystem, have the potential to exert their effects on our biological features and health status for most and perhaps all of our adult lives. This latter finding emphasizes the importance of understanding whether there is a definable program of community assembly in healthy infants/children and whether such a program is shared or varies considerably across populations with distinct dietary habits and culinary traditions residing in different geographic locations. If such a developmental program were definable and a significant contributor to healthy growth, fostering its proper and full execution could represent the basis of an arm of preventive medicine designed to ensure long-term health through informed microbial stewardship.

Food is a major factor that shapes the proportional representation of microorganisms present in the gut microbiota and the relative abundance of its genes (the microbiome). Reciprocally, the configuration of the microbiota/microbiome influences the nutritional value of food. One illustration of this interrelationship comes from a culture-independent metagenomic analysis of the gut microbiomes of infants, children, and adults belonging to 150 families living in three countries located on three different continents (metropolitan areas of the USA plus rural villages in southern Malawi and the Amazonas state of Venezuela). The results revealed that the relative abundances of genes in the microbiome that are related to vitamin biosynthesis (e.g., folate, cobalamin, thiamine, and biotin), amino acid metabolism, and processing of complex polysaccharides change in an identifiable sequence during the postnatal period (Yatsunenko et al., 2012). In addition, differences between Westernized (USA) and non-Westernized populations were evident, with breastfed Malawian and Amerindian babies having higher relative abundances of microbial genes encoding enzymes involved in carbohydrate metabolism, vitamin biosynthesis (e.g., components of the biosynthetic pathway for riboflavin, a component of breast milk, dairy products, and meat), and urease (Yatsunenko et al., 2012). Urea represents up to 15% of breast milk nitrogen; its degradation to ammonia can be used for microbial biosynthesis of essential amino acids, potentially benefiting both the microbiota and host when diets are deficient in protein. Significant differences in microbiome configuration were also observed between breast-fed and formula-fed infants, with the latter showing increased representation of genes involved in various aspects of carbohydrate and amino acid metabolism and cobalamin (vitamin B12) biosynthesis (Yatsunenko et al., 2012). Cobalamin is not only important for the host; the ability to transport cobalamin and other substituted corrins is an important determinant of survival for members of the microbiota (Degnan et al., 2014).

Together, these findings suggested that the gut community should be considered when assessing the nutritional requirements at different stages of the human life cycle and in different geographic/cultural settings. They also raised the question of whether perturbations in the functional development of the microbiota/microbiome were related to childhood undernutrition, the major cause of childhood deaths worldwide and a manifestation of a complex set of still poorly understood intra- and intergenerational factors, rather than food insecurity alone (Lazzerini et al., 2013; Caulfield et al., 2014; Richard et al., 2014).

Undernutrition and Gut Microbiota Immaturity

The World Health Organization's (WHO) Multi-Center Growth Reference Study (http://www.who.int/childgrowth/mgrs/en/) defines three anthropometric (physical) parameters (weight-for-age, height-for-age, and weight-for-height *Z* scores) to describe normal early childhood growth and nutritional status from its evaluation of 8,440 infants and children living in six distinct sites around the world (USA, Oman, Norway, Brazil, Ghana, and India). A recent study provided another definition of healthy growth but from a microbial perspective (Subramanian et al., 2014). It did so by examining gut microbiota assembly in 50 children residing in Dhaka, Bangladesh whose anthropometry during their first 2 years of life indicated healthy growth. Fecal samples were collected monthly from birth through the end of the second postnatal year, and the relative abundances of bacterial strains were analyzed by 16S rRNA amplicon sequencing. The results revealed that interpersonal variation in the bacterial component of their gut communities was significantly smaller than the variation associated with age. Applying Random Forests, a machine-learning method, to regress relative abundances of bacterial taxa across these children revealed age-discriminatory bacterial strains. Separating these 50 children into training and validation cohorts, the regression was optimized to include the most informative taxa for accurate prediction of microbiota "age." The results were formally validated to prevent over-fitting and over-estimation of generalizability and produced a sparse model composed of 24 strains that could be used in aggregate as a microbial signature for describing a shared program of microbiota development in healthy individuals and two derived metrics for defining deviations from that normal program: "relative microbiota maturity" and "microbiota-for-age" *Z* (MAZ) score (Figure 1).

Severe acute malnutrition (SAM) is defined by weight-for-height Z (WHZ) scores more than 3 SDs below the median of children in the WHO reference cohort. Application of this sparse model to 64 Bangladeshi children with SAM (WHZ -4.2 ± 0.72 [SD]) revealed they had gut microbiota that appeared significantly "younger" than their chronological age (relative microbiota maturity of -6 ± 0.7 months and MAZ scores of -1.7 ± 0.2). Moreover, this immaturity was incompletely and only transiently rescued following a customary period of administration of either one of two types of ready-to-use therapeutic foods (RUTFs; typically given for 2 weeks until a 15% increase in weight gain is achieved; http://www.ClinicalTrials.gov, number NCT01331044). Bangladeshi children with moderate acute malnutrition (WHZ between -3 and -2) also exhibited significant microbiota immaturity, although less severe than children with SAM (Subramanian et al., 2014). These results indicate that children with SAM have a persistent developmental abnormality

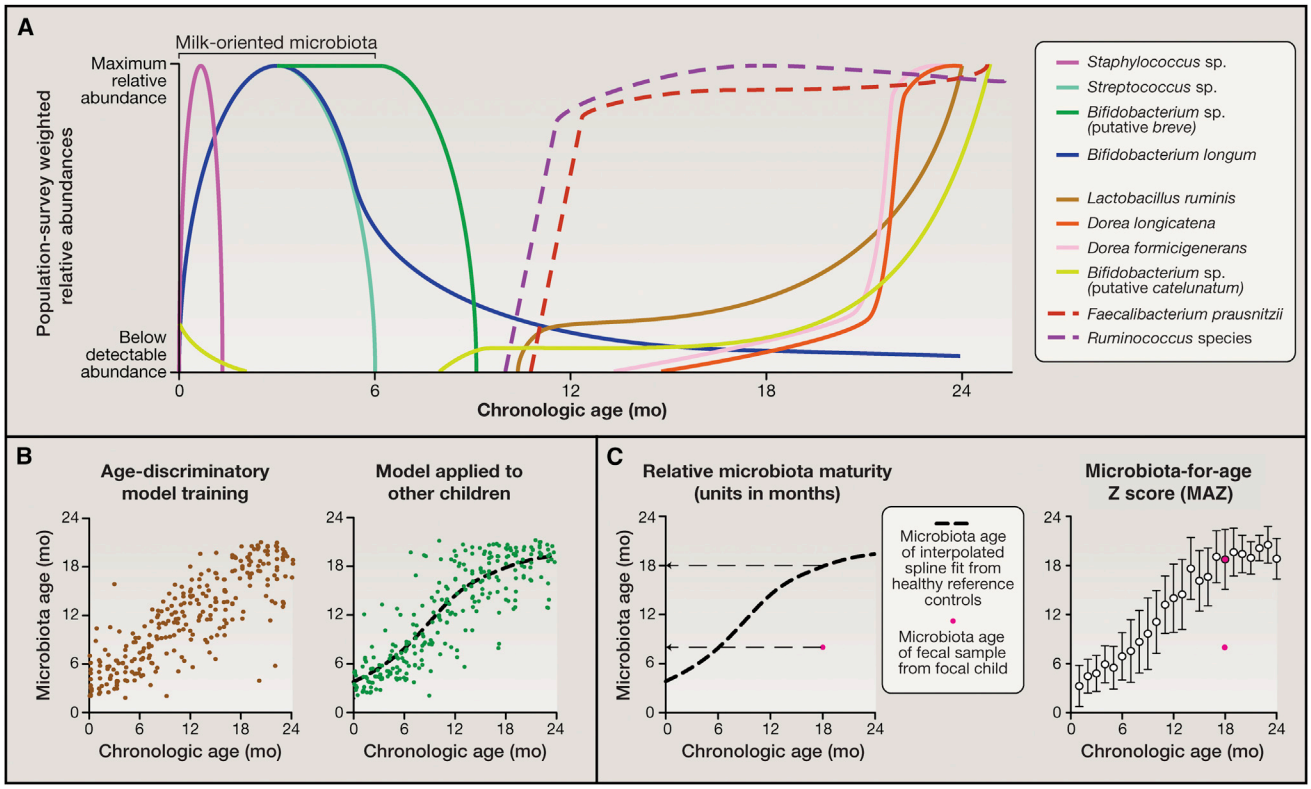

Figure 1. Developing Metrics for Describing Gut Microbial Community Development

(A) Bacterial taxa that discriminate different stages of development were identified by a machine learning-based (Random Forests) regression of 16S rRNA data sets produced from monthly fecal samples, collected from anthropometrically healthy infants and children living in an urban slum in Dhaka, Bangladesh during their first 2 years of postnatal life, to their respective chronologic ages at the time of sample collection (Subramanian et. al, 2014). Shown are depictions of the typical distributions of these age-discriminatory taxa across the population. Taxa were selected based on their relative importance to the accuracy of the Random Forests model using a permutation-based "feature importance." The y axis in the graph defines maximum relative abundance for each taxon in the microbiota in the context of the first 2 years of postnatal life.

(B) The most discriminatory taxa, as defined by their feature importance, were used as inputs into a sparse 24 taxon model whose output ("microbiota age") is a microbiota-based prediction of the chronologic age of a healthy child. The plot on the left of the panel shows microbiota age against chronologic age of healthy children used as a training set to fit the regression (each dot is a fecal sample from an individual child). The plot on the right of the panel shows application of the sparse model to a validation set composed of a different group of children living in the same location that were not used to train the model. Applying the model to a separate validation set controls for over-fitting of the model to the training set and ensures its wider usability.

(C) Two metrics of microbiota maturation based on application of the model to two separate validation sets of singletons and a separate study of Bangladeshi twins/triplets. "Relative microbiota maturity" is the deviation, in months, from a smooth-spline fit of microbiota age values with respect to chronologic age, fitted using the validation data sets (see black dashed curve). The red dot represents a fecal sample collected from a focal child that is 11 months below the spline fit, indicating negative relative microbiota maturity (i.e., an immature microbiota). MAZ is computed by dividing the difference between the focal child's microbiota age and the median microbiota age of healthy controls in the same monthly chronologic age bin over the SD within the same age bin. The median and SD of each bin are computed using the validation data sets. The distributions of microbiota maturity and MAZ scores in birth-cohort studies have been studied using linear mixed models that take into account random variation specific to each serially sampled child and family while estimating the fixed variation attributable to a factor observed across different children (e.g., diarrheal episodes) (Subramanian et al., 2014).

Note that using Random Forests to study microbiota maturation is advantageous because of its non-parametric assumptions and utility in the context of high dimensional data sets (large numbers of predictors). Nonetheless, it is one of several methods that can be useful. For example, the rank-order Spearman correlation metric has been applied to infant microbiome data sets to detect monotonic relationships between microbiome-encoded functions/bacterial taxa and postnatal age (Yatsunenko et. al, 2012).

affecting their gut microbial "organ" that is not durably repaired with existing therapy.

These observations raise a critical question: is microbiota immaturity a cause or an effect of childhood undernutrition? Many studies have shown that, although current protocols for treating children with (acute) undernutrition reduce mortality, they do not rescue its long-term morbidities, including stunting, immune dysfunction, and neurodevelopmental abnormalities (Victora et al., 2008, Gaayeb et al., 2014, Kosek et al., 2013, Galler et al., 2012). For example, given the remarkable metabolic requirements of the neonatal brain, alterations in the normal postnatal development of the gut microbiota may trigger marked impairments in brain development and lead to persistent disorders of cognition.

Support for a causal role for the gut microbiota in SAM comes from studies of gnotobiotic mice. In recent years, methods have been developed for transplanting previously frozen fecal samples from human donors into groups of germ-free mice at a selected stage of their lives (e.g., young, rapidly growing animals that have been recently weaned or older animals) and with a

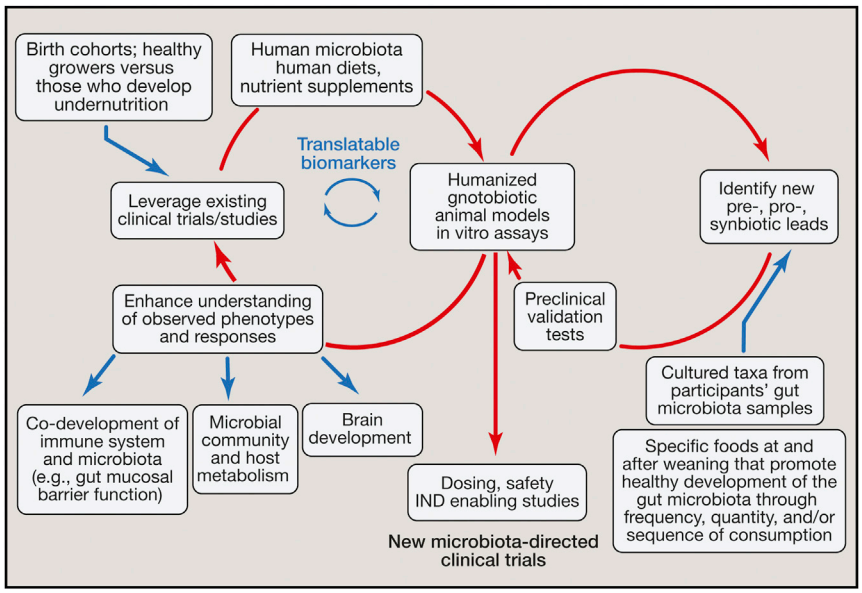

Figure 2. Integration of Existing Clinical Observational and Interventional Studies into Gnotobiotic Mouse Models to Identify Interactions between the Gut Microbiota, Food, and Host Biology

The discovery process depicted by the left circle illustrates how gnotobiotic animal models colonized with human donor microbiota and fed human diets can lead to a greater understanding of how diet-by-microbiota interactions are causally related to healthy growth and to phenotypes associated with undernutrition: e.g., immune system development, brain development, and host and microbial community metabolism. New surrogate- or mechanism-based biomarkers of nutritional state emanating from these gnotobiotic models can be validated using biospecimens collected from the donors used to construct these gnotobiotic models, as well as from other members of the study population. The discovery/development process depicted on the right illustrates how dietary and microbial "leads" can be tested in the context of humanized gnotobiotic animals to assess how they modulate biological processes already known, discovered, or postulated to be involved in healthy growth and/or the pathogenesis of undernutrition. The downward-pointing arrow in the middle of the figure points to next steps in clinical translation. See the main text for a discussion of the regulatory, ethical, societal, and commercial implications of these efforts. Abbreviation: IND, investigational new drug.

designated genetic background. If the human microbiota sample is frozen shortly after it is produced and maintained at −80°C, the bacterial strains represented in the donor's community can be transmitted efficiently and reproducibly to recipient mice (e.g., Turnbaugh et al., 2009a; Smith et al., 2013; Ridaura et al., 2013; Palm et al., 2014; Kau et al., 2015). The recipient mice can be fed diets that contain ingredients used in foods consumed by the microbiota donor. Moreover, the ingredients and methods for preparing (cooking) such diets can be varied systematically. This approach allows myriad types of models to be constructed for studying the interaction of foods and the human gut microbiota in vivo. For example, diets can be given that are representative of those consumed by populations other than those of the donor to anticipate the effects of changes in food consumption patterns associated with Westernization or composed of ingredients that represent new potential sources of affordable, nutritious foods such as landraces and waste streams from current food manufacturing processes. Critically, these preclinical gnotobiotic animal models allow proof-of-concept tests of whether a donor phenotype is transmissible via his/her gut microbiota, the extent to which phenotypic transmission generalizes across different donor microbiota, and the sensitivity or robustness of phenotypic transmission to diet type. These preclinical models also permit simulations of existing or anticipated therapeutic interventions, including the opportunity to "randomize" a given individual's microbiota to not just one but multiple treatment arms in order to directly compare the effect (and effect size) of the treatments on both the microbiota and host, to characterize underlying mechanisms, and to identify surrogate- or mechanism-based biomarkers that could be translatable to the microbiota donor or donor population (Figure 2).

Transplanting fecal microbiota from same-gender Malawian twins discordant for kwashiorkor, a form of SAM, into separate groups of adult germ-free mice and feeding the recipient animals a representative micro- and macronutrient-deficient Malawian diet disclosed that the healthy and kwashiorkor co-twins' microbiota transmitted discordant weight loss and metabolic phenotypes (as well as an enteropathy characterized by disruption of the small intestinal and colonic epithelial barrier in animals harboring kwashiorkor but not healthy microbiota) (Smith et al., 2013; Kau et al., 2015). Unlike the transplanted healthy co-twins' microbiota, the kwashiorkor microbiota was structurally and metabolically labile, reconfiguring itself upon exposure to a peanut-based RUTF, but not in a sustained way when animals were returned to the Malawian diet. The combination of a nutrient-deficient Malawian diet and a kwashiorkor microbiota was required to produce pathology in the recipient "humanized" mice, including inhibition of steps within the tricarboxylic acid cycle in host cells (Smith et al., 2013). These findings not only provided evidence for a causal relationship between the gut microbiota and SAM but also highlighted the importance of diet-by-microbiota interactions in disease pathogenesis.

If we consider children with persistent microbiota immaturity from the perspective of developmental biology, we can pose a number of basic and applied scientific questions. One question is whether the developmental program defined in Bangladeshi infants and children is generalizable to other populations representing different geographic and cultural settings. If so, it would reveal a fundamental shared aspect of postnatal human development and raise mechanistic questions about the factors that specify a healthy microbial community "fate." Initial support for generalizability comes from an analysis of concordant healthy Malawian twin pairs, which showed that a number of the age-discriminatory bacterial strains with the highest feature importance scores in the Bangladeshi Random Forests model are also represented in the Malawian population (Subramanian et al., 2014; Yatsunenko et al., 2012). The designation "same strain" was based on the same 16S rRNA sequence;

whole-genome sequencing of a given age-discriminatory strain identified by its 16S rRNA sequence will be needed to determine its degree of gene conservation across different Bangladeshi and Malawian hosts. Bacterial 16S rRNA analyses of fecal samples obtained at monthly intervals from infants and children with healthy growth phenotypes enrolled in birth cohorts living at multiple low-income countries allow country/community site-specific, Random-Forests-based models of microbiota maturation to be constructed, as well as an aggregate model representing data pooled from all sites. ''Generalizability'' can be established through reciprocal tests of the accuracy of the site-specific models (and aggregate model) for healthy individuals living at the different sites and whether these models reveal similar relationships between anthropometry and relative microbiota maturity/MAZ scores for undernourished children living at each of these sites.

A second question has to do with the relationship between microbiota development, enteropathogen load, and environmental enteric dysfunction (EED, also known as environmental enteropathy), an enigmatic and as-yet-incompletely defined disorder of gut barrier function (Keusch et al., 2014; Kosek et al., 2014). Does a primary failure to execute normal maturation of the microbiota directly influence risk for enteropathogen invasion, perturbations in development of mucosal immune system, and abnormalities in nutrient processing and absorption that ultimately results in growth faltering? Alternatively, is a holistic view required that considers each of these features of enteric biology as intimately and integrally related to one another? Large birth cohort studies such as MAL-ED and GEMS have provided an opportunity to measure the contributions of enteropathogen load/carriage and diarrheal incidence to growth faltering (MAL-ED Network Investigators, 2014; Platts-Mills et al., 2014; Kotloff et al., 2013). Evidence is emerging that some of the age-discriminatory taxa that define normal microbiota maturation also protect the host from enteropathogen infection. Intriguingly, studies of Bangladeshi adults with acute cholera have shown that recovery from the diarrheal phase involves recapitulation of the sequence of appearance of the same age-discriminatory bacterial strains that define the normal pattern of assembly of the microbiota in healthy Bangladeshi infants/children, suggesting that an essential set of rules governs this assembly (successional) process (Hsiao et al., 2014). For example, *Ruminococcus obeum*, a bacterium that directly correlates with recovery from *Vibrio cholerae* infection in adult Bangladeshi subjects and defines later stages of normal gut microbiota maturation in healthy Bangladeshi children, restricts *V. cholerae* colonization of gnotobiotic mice harboring a representative human gut microbiota. Its mechanism involves production of an autoinducer-2 (AI-2) that causes quorum-sensing mediated repression of *V. cholerae* colonization and virulence factor expression (Hsiao et al., 2014).

A third related question is the manner in which the mucosal immune system and the microbiota co-develop. How do these complex organs talk to and educate each other? The answers could help identify factors that legislate a normal developmental trajectory for a gut community and how developmental arrest of the microbiota could be become fixed and difficult to overcome/advance. Immaturity of the microbiota may be associated with relative immaturity of mucosal immunity in ways that impede responsiveness to vaccines or enteropathogens. If so, can we use members of the microbiota as next-generation adjuvants to prime the immune system in the context of a defined antigen (Yilmaz et al., 2014)? One way to characterize maturation of the mucosal immune system is to use fluorescence-activated cell sorting (FACS) to identify microbial taxa targeted by its IgA responses as a function of chronologic age in hosts with healthy growth phenotypes and in those with undernutrition (critically, IgA targeting is not simply a reflection of the abundances of organisms in the gut community; Kau et al., 2015). This method, named BugFACS, has identified bacterial targets of gut mucosal IgA responses using fecal samples from children with healthy growth phenotypes or those with varying degrees of undernutrition, as well as fecal samples harvested from gnotobiotic mice harboring transplanted microbiota from healthy and undernourished donors fed diets representative of those that these children consume. BugFACS-purified viable IgA-targeted bacterial taxa were subsequently introduced into germ-free animals fed nutrient-deficient or -sufficient diets to characterize their functional properties. The results disclosed that IgA responses to members of the microbiota can be used as biomarkers of growth faltering, that they are influenced by enteropathogen load, and that they mediate a diet-dependent enteropathy characterized by small intestinal and colonic epithelial barrier disruption. Moreover, treatment with IgA-targeted bacterial strains purified from healthy donor microbiota can prevent development of the enteropathy (Kau et al., 2015), indicating that this approach may have utility that extends beyond diagnostics to therapeutic lead discovery and defining mechanisms underlying EED pathogenesis.

A fourth and critical question is whether age-discriminatory taxa are not only just biomarkers but also effectors of growth. If so, they become potential therapeutic agents and targets for manipulation, including food-based manipulations that allow for their establishment in an individual or population at the time of presentation with manifest disease or prior to that time. One way we are currently determining whether age-indicative taxa are also growth indicative is by transplanting microbial communities from children exhibiting varying degrees of growth faltering (defined by anthropometry), representing a particular geographic region, into young, actively growing germ-free animals fed diets representative of the donor population and then defining the effects of the different transplanted communities on the growth, metabolic and immunologic phenotypes of recipient gnotobiotic mice (Figure 2). 16S rRNA data sets generated from the animals' fecal samples can be used to correlate strain abundances to these phenotypes. These strains can then be cultured from the microbiota of different donor populations. Determining the effects of subsequently introducing these strains—singly or as components of defined consortia—into young gnotobiotic mice harboring microbiota from different undernourished donors represents a way to address several challenges that would be faced when designing and interpreting a clinical study. For example, these preclinical studies could help to (1) define criteria used to select strains beyond their feature importance scores in the Random Forests models and cultivability (e.g., the extent of representation of virulence determinants in their genomes); (2) assess how to encapsulate these organisms, including anaerobes, in ways that permit their long-term storage and viability;

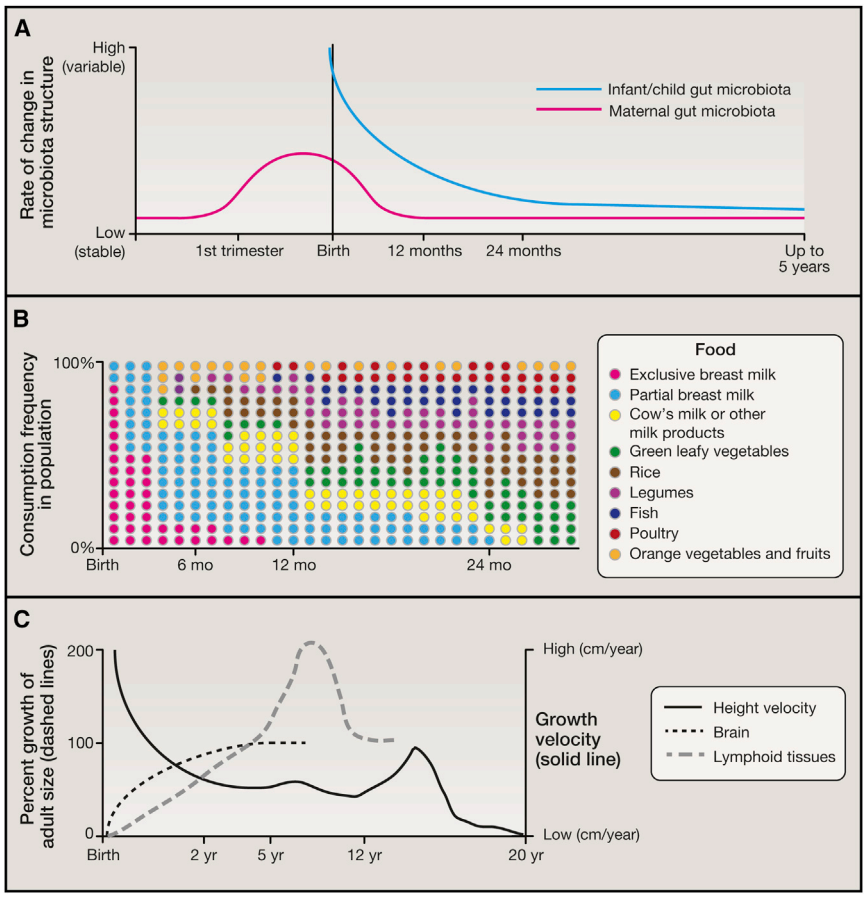

Figure 3. Co-variation in Gut Microbiota Assembly/Maturation, Dietary Patterns, and Other Facets of Human Postnatal Development

(A) Illustration of the rate of change occurring in gut microbiota structure of both mother and child. Note that infant variation curves are known from both longitudinal and cross-sectional study designs (Yatsunenko et al., 2012; Subramanian et al., 2014). In the case of mothers, the curve is interpolated based on studies of pregnant Finnish mothers prior to delivery (Koren et al., 2012) and Bangladeshi mothers following parturition (Subramanian et al., 2014). Note that the shape of the curve describing the evolution of the maternal microbiota during pregnancy has not been well defined and needs further clarification in multiple populations.

(B) The food consumption pattern shown is at a population level and does not depict the great deal of temporal variation observed in food consumption patterns within a given child. Depicting the fractional contribution of each food to the consumption patterns of children in Bangladesh underscores how dietary changes occur simultaneously (lowering of breast milk and increase in legumes and cow's milk) and not in an orderly fashion (small fluctuations from month to month; re-entry and dropout of certain foods). It also underscores the challenge encountered in ascertaining how food and the microbiota interact to effect maturation of the microbial community.

(C) Major processes related to growth and how they vary in rate and magnitude over time. Curves are adapted from Bogin (1999). Note that the newborn brain represents 12% of body weight (a value 6 times greater than in adults). By the end of the first decade, the brain represents 6% of body weight and consumes twice the amount of glucose and 1.5 times the amount of oxygen as the adult brain. Approximately 30% of the glucose consumed by the infant brain is accounted for by aerobic glycolysis (versus 12% in adults) (Goyal et al., 2014). The dramatic changes in brain metabolism that occur over the first two decades of life coincide with the initial proliferation and then pruning of synapses. Central questions that need to be addressed in this area include the biological effects of the gut microbial community on neurogenesis, synaptic connectivity, gliogenesis and glial-neuron interactions, neural circuit function and higher cognitive processes in the context of healthy growth versus undernutrition, and whether/how the gut-brain axis operates to influence/regulate other aspects of host physiology, metabolism, and immunity in the infant/child. Moreover, if persistent immaturity of the gut microbiota is causally related to undernutrition and its long-term sequelae, including neurodevelopmental abnormalities, does durable repair of this immaturity require that nutritional interventions be administered earlier before disease becomes fully manifest (and the microbial ecosystem is so perturbed that restoration becomes very difficult)? Do nutritional interventions need to be applied for more sustained periods of time? Do new types of therapeutic foods need to be developed, or is a microbial intervention also needed?

(3) determine the extent to which consortia can invade and establish themselves in different microbiota representing individuals from a given population or different populations; (4) assess the nature of their effects on growth (e.g., gain of lean body mass), metabolism, and gut barrier function as a function of the degree of donor undernutrition and microbiota immaturity; and (5) ascertain the degree to which invasion and establishment of these strains in the targeted microbiota and their host effects are impacted by diet. Determining whether these strains are interchangeable between countries will influence the generalizability of microbial interventions or whether there would have to be local sourcing of these biological resources by or for the communities who are themselves afflicted by undernutrition.

Establishing Microbiota and the Maternal Influence

The origins of the microbes that colonize an infant's gastrointestinal tract are complex, given that infants are exposed to different environmental sources. A major source is the mother and includes microbes from her vagina, skin, gut, and as some have reported, breast milk and possibly the placenta (Dominguez-Bello et al., 2010; Hunt et al., 2011; Grönlund et al., 2011; Cabrera-Rubio et al., 2012; Aagaard et al., 2014).

A key knowledge gap relates to the "anthropology of microbes": knowing how practices associated with pregnancy, including micronutrient supplementation, as well as traditional (and changing) societal "prescriptions" for dietary practices, impact a mother's microbial ecology prior to and following parturition and how this may impact transmission of her microbes to her infant. A study of 91 pregnant Finnish women showed that the maternal microbiota changes between the first and third trimester (Koren et al., 2012) (Figure 3). Another analysis of Bangladeshi mothers revealed marked changes in their gut microbiota in the first month post-partum, followed by less substantial changes in the ensuing 9 months (Subramanian et al., 2014). One testable hypothesis is that the maternal microbiota, much like the infant microbiota, undergoes stereotypical alterations during

normal pregnancy designed to enhance maternal health and to promote transfer of strains to the infant. Testing this hypothesis will require detailed time series sampling of maternal microbiota throughout pregnancy and of the maternal-infant dyad, plus other environmental sources, including other family members and caregivers. If a program of pregnancy-associated changes in the maternal gut microbiota can be identified using approaches analogous to those described above to characterize maturation of the infant microbiota, it could provide an opportunity to use the most indicative or transmissible taxa as biomarkers of nutritional status and as reporters of the effects of different dietary practices or the efficacy of prescribed prenatal nutritional interventions.

Pregnancy is also a time of increased susceptibility to infection. Rowe et al. (2011) demonstrated that pregnant mice show increased bacterial burden in models of *Listeria monocytogenes* and *Salmonella typhimurium* infection, mediated via active immune suppression by a population of FoxP3$^+$ regulatory T cells (Tregs). Moreover, ablation of the Treg compartment resulted in near-complete resorption of fetuses, indicating a delicate balance between immunological tolerance of the fetus and defense against enteropathogens (Rowe et al., 2011). It is not known how this period of deliberate immune suppression impacts the maternal microbiota and, in turn, transfer of pathogens (and other microbial community members) to the infant.

The Impact of First Foods

Breast Milk

The association between healthy postnatal growth and exclusive breastfeeding has led to the WHO's recommendation for a minimum of 6 months of exclusive breastfeeding (Kramer and Kakuma, 2002). Human milk is composed of lipids (tri-, di-, and monoglycerides, phospholipids, glycolipids, and free fatty acids), protein components (including immunoglobulins, lactoferrin, lysozyme, and cytokines), and a large repertoire of human milk oligosaccharides (HMOs). Over time, this composition changes from colostrum, which is HMO rich, to mature milk, which contains fewer HMOs and protein while the fat content remains relatively stable (Coppa et al., 1993; Lemons et al., 1982).

HMOs and other milk glycoconjugates pass undigested through the proximal gut (Engfer et al., 2000) and serve as nutrient substrates for saccharolytic microbiota in the colon. The microbiota of healthy exclusively breastfed infants is dominated by members of the genus *Bifidobacterium* (Figure 1; Yatsunenko et al., 2012; Subramanian et al., 2014). These infant-associated bifidobacteria, notably *Bifidobacterium longum* subsp. *infantis*, possess a suite of genes involved in importing complex fucosylated and sialylated milk glycans, their further degradation, and subsequent utilization (Sela et al., 2008). The functions encoded by this suite of genes allow them to outcompete other saccharolytic taxa (Marcobal et al., 2010). Bifidobacteria also actively reshape milk composition. For example, they release N-linked glycans conjugated to milk glycoproteins for use as a growth substrate. However, the effect of deglycosylation on milk protein digestibility and function is as-yet unknown (Garrido et al., 2012, 2013).

Colonization by *Bifidobacterium* species during nursing is associated with a range of benefits, including improved vaccine responses (Huda et al., 2014) and enhanced gut barrier function (Ewaschuk et al., 2008; Weng et al., 2014), including stabilized epithelial tight junctions noted in both animal models (Bergmann et al., 2013) and human cell lines (Chichlowski et al., 2012). Recent work has shown that infants with high *Bifidobacterium* population densities exhibit a corresponding decrease in fecal milk glycans (De Leoz et al., 2015; Wang et al., 2015), a relationship that could serve as the basis for developing inexpensive diagnostics to monitor development of a healthy gut microbiota in nursing infants.

Development of a healthy infant gut microbiota can be threatened by maternal undernutrition and premature birth. Maternal undernutrition during pregnancy increases risk for underweight and preterm births (Kramer et al., 1992). Children of undernourished mothers receive substantially less than the recommended intake of priority micronutrients during lactation (Allen, 2005). Fortified milk obtained from donors who have had a full-term pregnancy likely does not provide sufficient protein to preterm infants (Arslanoglu et al., 2009). Even when mothers of preterm infants can produce sufficient milk, alterations in milk fat, protein, oligosaccharide content (Weber et al., 2001; De Leoz et al., 2012), and the repertoire of immunoactive components (Castellote et al., 2011) are observed, leading to a call for identifying additional elements for nutritional support of these infants (Gabrielli et al., 2011; De Leoz et al., 2012).

A vicious cycle of maternal undernutrition and poor infant nutritional status can reflect alterations in the immune, HMO, and/or other components of mother's milk. This has critical implications for infant health. Poor maternal health is associated with variations in breast milk immunoglobulins and glycoprotein structures during lactation (Smilowitz et al., 2013) and with decreased lactoferrin, a protein with antimicrobial activities (Hennart et al., 1991). Parasite-specific breast milk IgA titers to *Entamoeba histolytica* and *Cryptosporidium* spp. correlate with nutritional status in a Bangladeshi infant population in which the burden of infection with these enteropathogens is very high (Korpe et al., 2013). Preterm delivery is associated with atypical variations in milk glycan structures (De Leoz et al., 2012), which poses additional risks. As HMOs have structural similarities to epithelial cell surface and mucus glycans, they can have anti-adhesive effects on enteropathogens. Sialic acid or fucose moieties are key determinants of this activity. Thus, variations in fucosylated HMOs associated with preterm birth may reduce the efficacy of milk oligosaccharides as anti-adhesive decoy molecules for pathogens (Ruiz-Palacios et al., 2003; Jantscher-Krenn et al., 2012).

Understanding how breast milk glycan repertoires correlate with normal microbiota assembly and with impaired microbiota maturation and undernutrition provides an opportunity to identify new glycan streams that could be used to treat undernourished infants. Commercial prebiotics are commonly added to infant formula, where they increase bifidobacteria titers in infant feces (Haarman and Knol, 2005; Knol et al., 2005; Boehm et al., 2002) and lower the incidence of pathogens (Knol et al., 2005). However, current prebiotics, namely fructooligosaccharides and galactooligosaccharides, do not represent the constellation of complex glycan structures delivered in human milk. Moreover, their consumption is not restricted to the population of microbes

that define normal gut microbiota maturation (Everard et al., 2014; Dewulf et al., 2013). Numerous efforts to recreate the glycan landscape present in human milk are underway. The technology for chemical and chemoenzymatic construction of complex "milk" oligosaccharides has advanced tremendously, enabling wholesale construction of a limited number of HMO-like structures present in milk (Muthana et al., 2009). Alternatively, purification from animal milks presents another opportunity for rapid and large-scale acquisition of milk oligosaccharides and glycoconjugates. At present, a number of enriched or purified bovine milk glycoproteins, including immunoglobins, lactoferrin, and glycomacropeptide, and glycolipids are commercially available or could be readily produced at scale for use in preclinical and clinical studies. Bovine milk contains a relatively low concentration of free oligosaccharides, but the distribution of structures observed roughly matches the most abundant species present in HMOs (Aldredge et al., 2013). Importantly, bovine milk oligosaccharides (BMOs) can be sourced from numerous points in dairy processing, including cheese whey, suggesting an opportunity for large-scale production of fractions enriched for given (or similar) structures (Zivkovic and Barile, 2011).

Serial Introduction of Complementary Foods in Ways that Promote Maturation of the Gut Microbiota

A recent study compared the microbiota and immune system in bottle-fed versus breastfed macaques. The results showed that breastfed infant macaques develop more robust T_H17 cells in the memory pool, suggesting that the timing and trajectory of dietary exposures during early life may have lasting functional consequences beyond that period (Ardeshir et al., 2014). In breastfed humans, the transition to formula feeding and family foods (complementary feeding practices) varies considerably in terms of which food types are consumed, the order of their presentation, and the duration of their consumption. Documenting which foods growing infants consume and in what quantities has required innovative approaches, particularly in low-income countries where undernutrition is prevalent (Caulfield et al., 2014) (Figure 3). For example, data collection protocols across eight different countries have been harmonized to enable quantification of variations in child feeding practices in the MAL-ED consortium (Caulfield et al., 2014).

The co-linearity between the introduction of various types of solid foods, reduction in breast milk consumption, and maturation of the gut microbiota makes it challenging to identify causal relationships between specific ingredients and the representation of specific microbes through human studies. However, studies in gnotobiotic mice colonized with defined collections of cultured (and sequenced) human gut-derived bacteria have been successful in interrogating specific food-microbe associations (Faith et. al., 2011). These relationships were identified using an experimental design in which a given gnotobiotic animal harboring a defined microbial consortium received a sequence of diets, composed of several different combinations of foods, whose concentrations are intentionally varied between diets. The order of presentation of the different diets was also varied between different mice in order to limit confounding from hysteresis effects. This approach has identified associations between various commercially available foods given in the USA during the complementary feeding period and specific microbes independent of their order of presentation, which would be virtually impossible to identify in clinical studies of developing human infants (Faith et al., 2011). This approach can be applied to young mice colonized with the age- and healthy growth-associated bacterial strains identified using the methods described above to determine which complementary foods promote their representation and expressed functional features. The results could lead to a recommended sequence of complementary feeding that reflects local food availability, affordability, and cultural practices and that sponsors healthy microbiota maturation. This information would advance current recommendations, which are not microbiota based and quite general (Kleinman, 2000).

Additional Considerations Regarding the Developmental Biology of the Gut Microbiota

Obesity

Although we have emphasized the global challenge of undernutrition in children, another vexing global health problem is the growing burden of obesity and associated metabolic dysfunction in children. Increasing attention is being paid to delineating differences in the gut microbiota of children who become obese in the hopes that early recognition of perturbed microbiota development may permit early interventions in at risk populations. For example, a recent culture-independent study of a Singaporean birth cohort disclosed that precocious maturation of the microbiota during the first 6 months of postnatal life was associated with significantly increased adiposity at 18 months (Dogra et al., 2015). Specifically, an unsupervised clustering approach based on bacterial 16S rRNA sequence data sets revealed three clusters of fecal microbiota configurations. The number of samples that binned into one of these clusters (cluster 3), which is characterized by high levels of Bifidobacteria and Collinsella and low levels of Streptococcus and Enterobacteriaceae, increased with age. A faster time to achieving a cluster 3 configuration was associated with significantly greater adiposity measured at age 18 months. Given the rapid rate of change in eating practices and incidence of childhood obesity, longitudinal studies of this type are timely. They should be strategically applied to populations representing different manifestations of these economic, anthropologic, and epidemiologic transitions and accompanied by comprehensive, quantitative assessments of food consumption during the pre-weaning, weaning, and postweaning periods.

Obesity is associated with reduced organismal and genetic diversity in the gut microbiota/microbiome of adults (Turnbaugh et al., 2009b; Le Chatelier et al., 2013). Transplantation of intact fecal microbiota samples, or derived culture collections, from adult twins stably discordant for obesity into germ-free mice transmitted the donors' discordant adiposity phenotypes, as well as obesity-associated metabolic dysfunction (Ridaura et al., 2013). Co-housing mice just after they received the obese donor's (Ob) microbiota with mice just after they received the lean co-twin's (Ln) microbiota, before their discordant adiposity/metabolic phenotypes became evident, prevented development of obesity and metabolic abnormalities in the Ob cagemate. This prevention was associated with unidirectional invasion of bacteria from the Ln cagemate's gut community to the

Ob cagemate's microbiota. Invasion was diet dependent, occurring in mice fed a human diet formulated to reflect the lower third of saturated fat and upper third of fruit and vegetable consumption in the USA, but not when animals received an unhealthy diet representing the upper third of saturated fat and lower third of fruit and vegetable consumption (Ridaura et al., 2013). These results illustrate how niches can be filled in the Ob microbiota by Ln-derived bacterial taxa to prevent disease and how important diet is to the installation of these health-promoting strains. The results raise important questions about the origins of the reduced bacterial diversity observed in Ob microbiota.

Impact of Antibiotics

One active area of investigation is the role of frequent consumption of broad-spectrum antibiotics in determining the diversity and functional features of the developing microbiota. Studies in conventionally raised mice treated with low-dose penicillin from birth to 4, 8, or 28 weeks of age revealed that early and brief exposure was sufficient to produce durable changes in body composition (Cox et al., 2014). Practical issues (in many parts of the world, antibiotic consumption in children is pervasive and poorly documented), ethical considerations, and the identification of suitable controls all confound the design of human studies that would seek to determine the effects of antibiotic administration on the developmental biology of the human infant gut microbiota and growth. In principle, pre-clinical tests that administer various classes of antibiotics in varying doses—together with representative human diets to gnotobiotic mice harboring transplanted microbiota from infants and children living in various parts of the world—followed by transplantation of their antibiotic-treated microbiota to a next generation of (antibiotic-free) gnotobiotic recipients, would provide one way to explore these questions.

Affordable Nutritious Foods: Societal Implications and Challenges

An imbalance of carbohydrate, fat, and protein consumption, food insecurity, and changing diets in low-income countries brought about by globalization, increases in food prices at the point of retail, and a global protein supply that needs to double by 2050 are some of the drivers for developing new types of affordable nutritious foods that are culturally acceptable, suitable for storage, and distributable given current and envisioned future infrastructure. A sustainable economic model in which local economies benefit from producing and/or distributing foods is also required to ensure long-term supplies. Moreover, there is a paucity of generally accepted metrics for defining foods that provide optimal nutrition at affordable cost (e.g., see the "nutrient-rich foods index" developed based on FDA recommendations; Drewnowski, 2010).

We propose that the gut microbiota provides a parameter that needs to be considered when developing nutrition options and that the type of preclinical gnotobiotic models described above will be useful for testing and defining dietary parameters. Studies with mice and other species provide means for characterizing interactions between food ingredients (at different levels of ingredient resolution and including culturally relevant spices and sweeteners), their methods of preparation and preservation, the gut microbiota of various consumer populations, and human metabolic, immunologic, and other physiologic features. These research platforms offer the promise of yielding next-generation foods designed to be satiating, delicious, nutritious, and able to manipulate microbiota and host properties in ways that promote healthy growth and wellness. However, fulfilling this promise demands a holistic view of the nexus of human gut microbial ecology research, agricultural practices, food production, evolving consumer tastes in an era of rapid globalization, envisioned commercialization strategies, current regulatory structures/practices, ethical issues, and public education. For example, there is a need to more thoroughly and rapidly characterize, through readily searchable, accessible, well-annotated databases, emerging food consumption patterns in countries representing different cultural traditions, stages of economic development, and land/water resources. At the commercial level, there is an opportunity to define and differentiate foods based on their effects on different consumer populations with distinct biological phenotypes and with different gut microbial community configurations. There is an accompanying need to frame intellectual property laws in ways that provide appropriate incentives for private investment while protecting the public good.

To effectively and responsibly apply this knowledge in ways that benefit society, there is a need to work with government agencies to provide efficient and sensible regulatory schemes. These regulatory frameworks vary between nations and are evolving. Currently, the US Food and Drug Administration (FDA) defines "medical foods" as foods that make medical claims. A "dietary supplement" is a product intended for ingestion that contains a dietary ingredient designed to add further nutritional value to a diet. Dietary supplements can only contain ingredients that are "generally regarded as safe" (GRAS) or approved as food additives by the FDA after filing a "new dietary ingredient" (NDI) notification with full description of the ingredient and product in which it will be marketed, the basis for the manufacturer's conclusion that it is an NDI, recommended use and proposed labeling, plus a history of its use and evidence of its safety to support the proposed use. Probiotics have been defined in various ways, including "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics, 2001), whereas prebiotics have been considered to be "a selectively fermented ingredient that allows specific changes both in the composition and/or activity of the gastrointestinal microbiota that confer benefits upon host well-being and health" (Roberfroid, 2007). Synbiotics are combinations of prebiotics and probiotics. Regulation of prebiotics, probiotics, and synbiotics remains a work in progress, although any health claims they make will likely require a clinical development pathway that is the same as that employed for biologics.

Opening the Public Discussion

For public acceptance and societal benefit, a thoughtful proactive, science-based, educational outreach is needed with an understandable vocabulary tailored to targeted consumer populations and respectful of their cultural traditions. The goal would be to objectively describe the extent to which the nutritional

value of food is related to a consumer's microbiota and how food ingredients, food choices, and the microbiota are connected to health benefits.

We suggest that one way of framing a public discussion regarding the impact of human gut microbiome research on the nexus of food, agriculture, and nutrition is to divide it into three "sectors": science and technology, ethics, and policy and governance.

Science and Technology

Ongoing and new studies will help to define (1) methods for selection and production of new food sources, (2) design of new foods/diets, (3) definitions of nutritional value and benefit and metrics for differentiation of foods, and (4) the role of the gut microbiota in determining nutritional status in pregnant women, infants and children, and adults throughout the course of their lives.

Ethics

The impact of gut microbiota research extends beyond conceptions of health to human rights. Key issues include (1) concepts of self and ownership of microbes and the shaping of these views by cultural, religious, socio-economic, educational, and political factors; (2) use of a person's microbes to improve nutritional status within and beyond family, community, and country; (3) strategies for responsible stewardship of our (human) microbial resources; and (4) personal, familial, and societal impact (and shared benefit) of methods envisioned to promote intergenerational transmission of beneficial microbes and to effect durable repair of defective gut microbial community development early in life or functional restoration later in life.

Policy and Governance

Advances in gut microbiota research will have long-term impact on regulatory and other governmental policies and agencies as they relate to agriculture, food, and nutritional health. These effects include (1) definitions of food safety, including the products of microbial biotransformation of food ingredients; (2) definitions of nutritional benefit within and outside of the context of specific human health claims; (3) laws concerning ownership of microbial strains and their distribution within and across national borders (for example, in October 2014, the *Convention on Biological Diversity/Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits from their Utilization* entered into international force "stringent requirements for prior informed consent and benefit sharing for research and commercial activities involving genetic resources from plants, animals, and microorganisms" [http://www.cbd.int/abs/]); (4) laws concerning intellectual property related to microbes, microbial consortia, and the products of microbial interactions with food ingredients, including diagnostics and therapeutics; (5) policies related to standards of manufacture, purity, and composition of probiotics and synbiotics; and (6) incentives for linking plans for food production and distribution with gut microbiota health. A key challenge is how to construe (1)–(6) in the context of a reference set of "representative" countries.

Closing Thoughts

Given the intricate links between first foods and long-term human health, ensuring availability of appropriate food sources is of high priority. Because undernutrition is such a widespread affliction, it is critical to consider how to categorize the targeted populations, the cost and economic sustainability, the efficacy (effect size and durability), and the cultural acceptability of various therapeutic or preventative approaches, as well as the generalizability of both food-based and microbial interventions to large populations within and across national/societal boundaries. One way of conceptualizing this complex set of challenges for treatment and prevention is to place, on one end of the spectrum of undernutrition, children with already manifest SAM and significant microbiota immaturity who could be treated with locally produced, readily and reproducibly manufactured, affordable and safe, culturally acceptable next-generation RUTFs, with or without microbial interventions of the type described above. Moving along this continuum, another group would consist of individuals who manifest growth faltering (stunting) in the first 1,000 days after conception, where the envisioned targets for interventions are pregnant and lactating women and their infants. At the other end of the continuum is a third group that are the targets of locally produced, consumer-focused, affordable nutrition products designed to improve dietary quality and increase the diversity of food choices.

Looking back over 800 million years of metazoan evolution, we appreciate more now than ever before the splendid innovation of having a gut that assembles microbial resources that enable efficient utilization of available nutrients (McFall-Ngai et al., 2013). We, humans, are now in a position to not only understand but to deliberately influence this process of microbial community acquisition in order to ensure its optimal execution. The challenges we face in designing and improving food systems and nutritional health are great and pressing. Hopefully, our gut instinct will be to honor and harness the intimate interrelationship between foods and "our" microbes in an attempt to address this challenge now and throughout the course of this defining century for our species and planet.

ACKNOWLEDGMENTS

Work cited from the authors' labs was supported in part by grants from the Bill & Melinda Gates Foundation (BMGF) and the NIH (DK078669, DK30292, and DK70977). We thank our colleagues in the BMGF-sponsored Breast Milk, Gut Microbiome, and Immunity Project, Tahmeed Ahmed and other members of our collaboration with the International Center for Diarrheal Disease Research, Bangladesh (ICDDR,B), Michael Barratt, Jeanette Gehrig, Siddarth Venkatesh and other members of our laboratories, plus William Petri and Andrew Serazin for their insights and inspiration. J.I.G. is a co-founder of Matatu, Inc., a company characterizing the role of diet-by-microbiota interactions in animal health. D.A.M. is a co-founder and President of Evolve Biosystems, Inc., a company focused on diet-based manipulation of the gut microbiota.

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Leading Edge
Review

Cell

Fermented Foods as Experimentally Tractable Microbial Ecosystems

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http://dx.doi.org/10.1016/j.cell.2015.02.034

Microbial communities of fermented foods have provided humans with tools for preservation and flavor development for thousands of years. These simple, reproducible, accessible, culturable, and easy-to-manipulate systems also provide opportunities for dissecting the mechanisms of microbial community formation. Fermented foods can be valuable models for processes in less tractable microbiota.

Introduction

The study of microbial communities currently faces a difficult impasse. As we continue to amass terabytes of sequencing data that describe the phylogenetic diversity of microbes around the world, we are faced with the challenge of dissecting the assembly, organization, and functions of these multi-species communities. Major questions remain about the nature, extent, mechanisms, and impact of species-species and species-environment interactions within microbial communities.

There are a number of barriers to understanding microbial ecosystems. First and foremost is the enormous diversity and complexity of most microbial communities. DNA sequencing-based surveys have now been applied to many habitats and provide a picture of the microbial diversity within and across environments (Lozupone and Knight, 2007). Although there is substantial variation from one environment to the next, the number of species in most habitats can reach into the hundreds or thousands. The inability to isolate the vast majority of species from natural systems by culturing is another significant barrier to characterizing the role of any given species in an ecosystem. A related constraint is the difficulty in recreating experimental conditions in an in vitro setting such that processes can be studied under controlled conditions.

One way to overcome these challenges is to move toward the development and analysis of simpler and experimentally tractable model microbial communities. An ideal model system should be simpler than natural communities yet still exhibit patterns of community formation and dynamics that are representative of those observed in more complex systems. The microbial communities involved should form under defined, reproducible, and easily accessible spatial and temporal scales to allow for predictable and straight-forward sampling procedures. The conditions for community formation and substrates of microbial growth should be measurable and possible to recreate in an in vitro setting. Finally, the individual members of the community should be culturable to facilitate application of the full range of genetic and omics-enabled tools, experimental analysis, and in vitro community manipulation.

Model microbial ecosystems have already emerged that have some of these properties (Jessup et al., 2004). These systems range from synthetic mixtures of model microbial strains (Harcombe et al., 2014; Hom and Murray, 2014), to model systems composed of a subset of culturable strains in complex free-living or host-associated communities (Lawrence et al., 2012; Peay et al., 2012; Goodman et al., 2011), to naturally occurring communities that are intensively sampled or perturbed in situ (Tyson et al., 2004). Studies across this spectrum have provided incredible opportunities to dissect the biology of microbial communities. However, systems that fall in the middle of the spectrum of simplified synthetic to irreducibly complex are needed help us to move toward a mechanistic understanding of microbial communities.

Fermented Foods as Experimentally Tractable Ecosystems

Fermentation is an ancient method for preserving foods that depends on reproducible formation of multi-species microbial communities. Over thousands of years, humans have optimized the conditions that promote the growth of certain types of microbial communities. The metabolic activity of these communities is key to the safety, flavor, texture, and aroma of fermented foods (Hutkins, 2006). Manipulation of microbial growth variables such as temperature, salinity, and moisture results in a wide spectrum of fermented foods, including cheese, beer, wine, chocolate, sourdough, sauerkraut, kimchi, and miso (Figure 1 and Table 1). The distinct microbial communities involved in fermenting these foods have a number of properties that make them ideal candidates for development as experimentally tractable model ecosystems.

Because the microbial communities of fermented foods (MCoFFs) offer a wide range of paradigms for community formation, it is possible to address experimental questions across many different types of ecosystems (Table 1). These communities take many forms: multi-species biofilms associated with surfaces (e.g., cheese rinds), suspended biofilms in liquid (e.g., kombucha, kefir, and vinegar), dispersed growth in liquid (e.g., lambic beers, natural wines, and yogurt), or in semi-solid substrates (e.g., kimchi and miso).

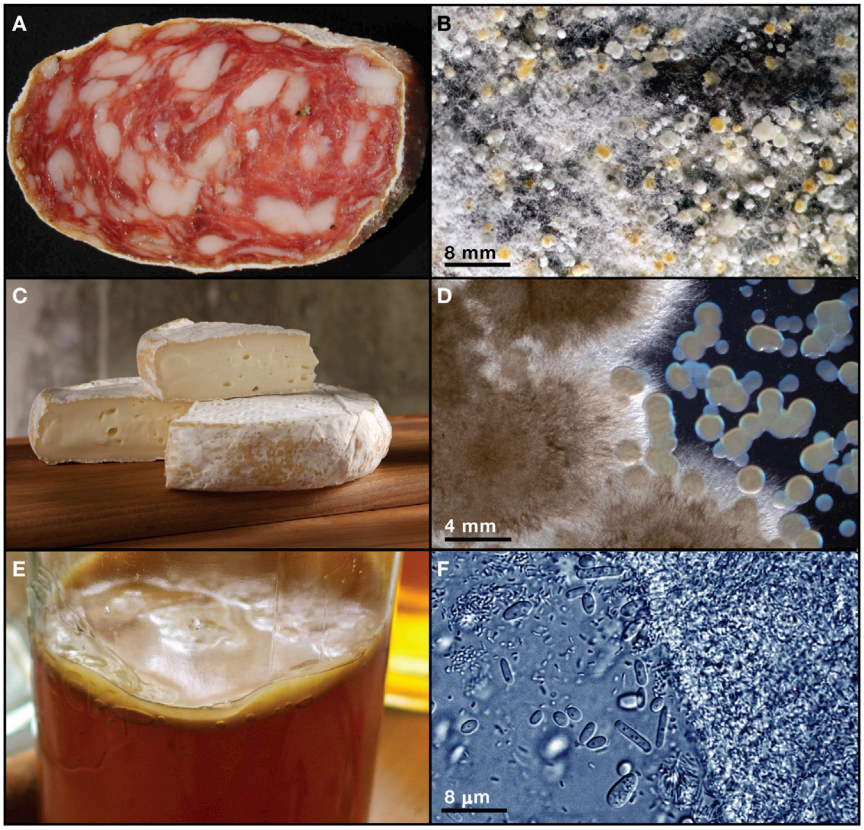

Figure 1. Multi-species Microbial Communities Form during the Production of Fermented Foods

(A) Fermented meats, such as salami, are produced by fermentation of meat by lactic acid bacteria.

(B) During the aging process, the salami surface is colonized by a mixture of yeast and bacteria, visible as white and yellow colonies, and filamentous fungi (diffuse white filaments) such as *Penicillium*.

(C) Cheeses, such as the Camembert-style cheese shown, are made through the fermentation of milk by lactic acid bacteria. During aging, a biofilm, commonly called a rind, develops on the surface and contributes to the flavor, texture, and aroma of the cheese.

(D) A rind biofilm plated on standard lab medium shows a subset of the mixed eukaryotic (filamentous fungi on the left) and prokaryotic (Proteobacteria on the right) members of these microbial communities.

(E and F) (E) Visible microbial communities also form in liquid fermentations, such as this fermented tea, commonly known as kombucha. The microbial cells within the pellicle (floating biofilm) can be seen in the micrograph (F). Kombucha is typically composed of yeasts (larger cells) and acetic acid bacteria (smaller cells). The yeasts are involved in the fermentation of sugar to produce ethanol and carbon dioxide. The acetic acid bacteria then ferment the ethanol and produce acetic acid. The intact biofilm is on the right, and yeast and bacterial cells are sloughing off on the left.

All photos by Benjamin Wolfe, except (C) (Jasper Hill Farm) and (E) (Adam DeTour).

Recent advances in genomic and metagenomic sequencing are providing researchers with catalogs of the bacterial, fungal, and viral diversity in many traditionally produced fermented foods (Table 1; reviewed in Bokulich and Mills, 2012). These communities range in composition from those dominated by bacterial species to those dominated by fungal species, with some communities containing a mix of both bacteria and fungi. Certain bacterial groups such as the lactic acid bacteria (LAB) and acetic acid bacteria (AAB), as well as fungal species such as *Saccharomyces cerevisae*, have well-established roles in fermentation. However, the increasingly detailed analysis of the microbial diversity of fermented foods is revealing many additional species whose roles have not been characterized extensively, if at all. For example, marine-associated *Pseudoalteromonas* are dominant members of some cheese rinds (Wolfe et al., 2014) (in Table 1, these and all non-LAB/AAB fall under "other bacterial groups").

After characterizing the diversity of a microbial community, one of the biggest challenges in the study of microbial ecosystems is the difficulty in culturing community members in the laboratory. Because MCoFFs have defined starting materials as growth substrates (e.g., milk, grapes, and wheat flour) and known incubation conditions, these same conditions can be replicated in the lab and used as starting conditions for isolation of community members. Indeed, some food-associated microbes are already well-established model organisms, such as *Saccharomyces cerevisiae* and *Lactococcus lactis*.

Experimentation using MCoFFs is also greatly facilitated by the fact that they are extremely accessible microbial ecosystems. The production of fermented foods happens at regular intervals (from daily to seasonally), and communities develop on short timescales (from days to months), allowing for predictable access to many replicated samples over relatively short time periods. Fermented foods are often produced across multiple geographic regions, also increasing the accessibility of samples. These communities form as part of discrete entities (e.g., a wheel of cheese), which allows well-defined spatial and temporal sampling.

MCoFFs have some potential limitations as model systems. Given the short timescales required to form communities, there may be fewer opportunities for species to coevolve. However, horizontal gene transfer between species that co-occur in MCoFFs suggests that at least some members of these communities have coexisted long enough to allow for gene exchange (Cheeseman et al., 2014; Rossi et al., 2014). In fact, some MCoFFs are maintained for many years through serial transfer (Table 1), providing ample opportunities for long-term coevolution within communities. For example, fermented teas such as kombucha consist of a pellicle that contains bacteria and yeasts in a mixed biofilm (Figures 1E and 1F). These pellicles have been spread all around the world (Marsh et al., 2014), leading to geographically separated communities that potentially started from initially identical species and genetic backgrounds.

Because MCoFFs grow on raw food materials, such as grains, meat, or milk, most nutrients are not limited. This high resource

Table 1. Diverse Fermented Foods Provide Ample Opportunities to Study Microbial Communities

Type of Food	Fermented Product	Main Ingredients	Major Microbial Groups	Opportunities to Study	References
Fruit	wine	pressed grapes	Y (natural styles: LAB)	biogeography, population biology due to wide geographical distribution	Bokulich et al. (2014a); Knight and Goddard (2015)
	chocolate	cacao pods	FF, Y, LAB, AAB, OBG	community interactions and dynamics due to successional development and broad phylogenetic diversity	Meersman et al. (2013)
	coffee	coffee cherries	FF, Y, OBG	community interactions and dynamics due to successional development and broad phylogenetic diversity	Vilela et al. (2010)
Dairy	yogurt	milk	LAB	co-evolution and adaptation due to serial transfer over long time periods	Sieuwerts et al. (2008)
	cheese	milk, salt	FF, Y, LAB, AAB, OBG	biogeography, community interactions and dynamics, and abiotic selection due to wide geographical distribution, broad phylogenetic diversity, and strong abiotic filters	Wolfe et al. (2014); Montel et al. (2014)
	kefir	milk	Y, LAB, AAB, OBG	co-evolution, adaptation, and biofilm formation due to self-replicating, highly organized biofilm and serial transfer over long time periods	Marsh et al. (2013)
Grains	beer	barley, hops, water	Y (lambic styles: AAB, LAB, OBG)	adaptation, community interactions and dynamics in lambic styles: accumulation of species in facility, successional development	Bokulich et al. (2012)
	sake, soy sauce, miso	rice, water (soy beans added for soy sauce and miso)	FF, Y, LAB, AAB, OBG	community interactions and dynamics due to successional development, adaptation due to domestication of Aspergillus oryzae	Bokulich et al. (2014b); Gibbons et al. (2012)
	sourdough	wheat flour, water	Y, LAB	biogeography, co-evolution, adaptation due to wide geographical distribution and serial transfer over long time periods	Minervini et al. (2014)
Meat	salami	ground meat, salt	FF, Y, LAB, OBG	community interactions and dynamics due to broad phylogenetic diversity	Cocolin et al. (2011)
Plants	kimchi	cabbage, spices, salt	Y, LAB	community interactions and dynamics, abiotic selection due to successional development and strong abiotic filters	Jung et al. (2011)
	sauerkraut	cabbage, salt	LAB	community interactions and dynamics, abiotic selection due to successional development, abiotic filters	Plengvidhya et al. (2007)
	kombucha	tea, sugar	Y, LAB, AAB, OBG	co-evolution, adaptation, biofilm formation due to self-replicating, highly organized biofilm and serial transfer over long time periods	Marsh et al. (2014)
				Key: FF = filamentous fungi; Y = yeast; LAB = lactic acid bacteria; AAB = acetic acid bacteria; OBG = other bacterial groups	

availability may be one reason why productivity is high in these systems and diversity can be low (Mittelbach et al., 2001). However, certain micronutrients such as iron, which has been shown to be a crucial factor in microbial interactions and coevolution in other environments (Cordero et al., 2012), is also limited in some MCoFFs, such as cheese rinds (Monnet et al., 2012). Finally, MCoFFs are not host-associated communities. Although this may simplify many aspects of experimental analysis, the impact of host innate and adaptive immune responses in dictating microbial community composition will be missing. An exception is the presence of biologically active host-derived products in milk, such as lactoferrin, oligosaccharides, and peptides (German et al., 2002).

The development of model systems based on fermented foods is already in progress. For a number of systems, the catalogs of microbial diversity within and across foods, temporal dynamics of community formation, and cultured community members are already established (Table 1). As an example of the characterization and development of such a system, we have recently focused on the microbial communities that form on the surface of cheese as it ages, also known as the rind (Figures 1C and D) (Wolfe et al., 2014). We cataloged the diversity of bacteria and fungi of cheese rinds across broad geographic regions, and measured the temporal dynamics of community formation. We cultured representatives of all dominant microbial groups and then reconstructed in vitro communities, which displayed many of the properties of natural communities. The ability to move quickly from observations of microbial diversity to the establishment of highly manipulable in vitro systems makes model systems based on MCoFFs excellent starting points for studying the mechanisms and principles of microbial community formation.

Using Fermented Foods to Link Patterns, Processes, and Mechanisms of Microbial Community Assembly

One of the current challenges in microbiology is linking patterns of microbial diversity within communities with the ecological processes that generate those patterns. What determines the composition of a microbial community? When and how do new species successfully invade communities? What causes shifts in composition of established microbial communities? To address such questions, microbiologists have begun to adopt community assembly frameworks developed for plant and animal communities to explain ecological processes underlying patterns of diversity (Costello et al., 2012; Hanson et al., 2012; Nemergut et al., 2013). Community assembly approaches usually consider contributions from the amount and timing of microbial propagules colonizing a habitat (dispersal), interactions between species (biotic selection), interactions between a species and the environment (abiotic selection), stochastic changes in the relative abundances of species within communities (drift), and evolution of new species within communities (diversification).

MCoFFs provide many opportunities for quantifying the relative roles of each of these ecological processes because microbial community dynamics can be easily measured, monitored, and controlled. For example, dispersal can be manipulated by controlling the openness of system (e.g. by simulating a highly controlled and sterilized environment versus a rustic production facility open to migration), using pasteurization of raw materials, or by incorporating known starter cultures. Biotic selection can be manipulated through the addition of specific combinations of species that are known to have strong species interactions. Abiotic selection can be controlled through the same selections that food producers use, including the manipulation of salt, moisture, temperature, and pH. These systems offer the ability to alter inputs and known ecological filters and allow testing of the relative impacts of each process on the composition of communities.

MCoFFs are particularly ripe with opportunities to link pattern, process, and mechanism in the study of microbial interactions (Mounier et al., 2008; Sieuwerts et al., 2008). For example, most fermented foods go through a clear and relatively consistent process of ecological succession with early colonizing microbes being replaced with one or more succeeding microbial groups. In some systems, such as the fermentation of cocoa pods (Meersman et al., 2013) and sourdough fermentations (Minervini et al., 2014), changes in the environment caused by early colonizing species and metabolic cooperation between functional groups are underlying drivers of these successions.

In vitro community reconstructions and the ever-growing suite of microbial "-omics" tools can help uncover the molecular mechanisms driving succession and identify how species interactions play a role in these temporal changes in community composition. Approaches such as RNA-seq can be used to winnow many potential mechanisms down to a short list of molecular mechanisms underlying microbial interactions. The application of sequencing-enabled transposon mutagenesis (TnSeq and INseq) has been successful in elucidating key microbial interactions in complex microbial communities such as the human gut microbiome (Goodman et al., 2009) and could be an extremely powerful tool for the analysis of microbial interactions in MCoFFs. In addition, new approaches in the identification of molecules responsible for species interactions, such as imaging mass spectrometry (Watrous and Dorrestein, 2011), could enrich our understanding of the chemical cross-talk in MCoFFs.

Taste of Place? Microbial Biogeography of Fermented Foods

Similar types of MCoFFs are produced in many locations around the world (Table 1), making it possible to address basic questions in microbial biogeography. Because microbial diversity can have an important impact on the flavor of fermented foods, such as wine or cheese (Ciani et al., 2010; Montel et al., 2014), MCoFFs can help link patterns in microbial diversity from place to place to the functional consequences of this diversity. As with plant and animal communities, microbial communities have clear species abundance distributions, with a few microbial taxa that are locally abundant within and across communities, while many tend to be relatively low abundance (Nemergut et al., 2013). This pattern has also been observed in high-throughput sequencing surveys of MCoFFs (e.g., Jung et al., 2011; Wolfe et al., 2014), although the typical long tail of low-abundance species tends to be shorter due to the lower diversity in MCoFFs. Thus, MCoFFs can be used to compare the patterns of community diversity over both regional scales, such as in wine

(Bokulich et al., 2014a), and over global scales, such as with cheese (Wolfe et al., 2014).

Trait-based approaches have also emerged to address questions of microbial abundance across these larger spatial scales. Trait-based approaches attempt to use either directly measured microbial life-history traits, such as growth rate or predicted traits from genomic data (Fierer et al., 2014), to explain distributions or functions of microbial species. Unlike many microbial communities dominated by unculturable taxa, it is fairly straightforward to measure phenotypic and genomic traits of species within MCoFFs (Bayjanov et al., 2013; Almeida et al., 2014; Douillard and de Vos, 2014). MCoFFs that are open to dispersal, widely distributed, and are not heavily inoculated with starter cultures, including naturally fermented wines, unpasteurized rind cheeses, and sourdough breads (Table 1), could be ideal MCoFFs for linking the genetic basis of interspecific trait variation with geographic distributions.

Microbial Evolution in a Community Context

MCoFFs have ideal properties for dissecting ecological processes, but they can also serve as models for understanding mechanisms of microbial evolution. Beer and wine yeasts, lactic acid bacteria used in dairy fermentations, and the filamentous fungus *Aspergillus oryzae* used to make sake, soy sauce, and miso have all served as models of microbial domestication (Douglas and Klaenhammer, 2010; Gibbons et al., 2012; Steensels and Verstrepen, 2014). Comparisons of genomic and phenotypic traits across the large diversity of fermentation and wild strains provided clear evidence for consistent loss of genes unnecessary in the nutrient-rich fermentation environment, acquisition of new traits through horizontal gene transfer, and metabolic remodeling associated with adaptation to the fermentation niche and artificial selection by humans. Experimental evolution of wild strains to the fermentation environment has been used to confirm potential genetic mechanisms involved with the transition to domestication (Bachmann et al., 2012).

One exciting future direction is to link ecological processes described above with the study of evolutionary processes in MCoFFs. Recent work from other model microbial ecosystems has highlighted the importance of eco-evolutionary feedbacks, where the presence and composition of neighboring species can alter evolution of community-level traits, such as species composition (Celiker and Core, 2014), and ecosystem-level traits, such as productivity (Lawrence et al., 2012). MCoFFs that have experienced long periods of serial passage or co-culture could serve as powerful model systems in this emerging field. Future studies could experimentally test for coevolution within these communities by swapping identical species from across geographically isolated communities. Applying experimental evolution approaches in the lab would allow for real-time monitoring of community coevolution within MCoFFs.

Translation to Other Microbial Communities and Potential Applications

The study of community assembly in MCoFFs has the potential to have direct impacts on the quality and safety of traditional fermented foods. But how might basic ecological and evolutionary principles discovered in these semi-natural microbial ecosystems be applied to other types of microbial communities such as the human microbiome or soil microbial communities? Certain MCoFFs share compositional similarity with less tractable systems, so pattern-process-mechanism relationships identified in MCoFFs may be readily translatable to other microbiomes. One example is the recently characterized molecular mechanism of competitiveness in *Lactobacillus reuteri*, a species found in both sourdough and human gut microbiomes (Lin and Gänzle, 2014). In sourdough fermentations, glycerol metabolism explained the competitive advantage of human-associated strains and potentially could explain how these species compete in human gut microbiomes.

Looking at cheese rind microbial community diversity revealed a diversity of bacteria and fungi with similarity at the genus level to the human skin microbiome (Wolfe et al., 2014). Data from both cheese rinds and the human skin microbiome (Grice et al., 2009) suggest that moisture is a major driver of these surface biofilms, suggesting that ecological selection could play similar roles in both. Thus, although the exact species may not be the same between MCoFFs and other microbial communities, community-level processes will likely be conserved.

MCoFFs have the potential to directly impact human health because they are edible communities. Although the probiotic effects of highly simplified MCoFFs from yogurt have been the topic of intense study (McNulty et al., 2001), the wider microbial diversity across MCoFFs could be a source of many additional microbes and metabolites with direct access to the human digestive tract and gut microbiome. The potential for direct interaction with the human microbiome is evidenced by the findings that MCoFFs can remain viable during passage through the human digestive tract (David et al., 2014).

MCoFFs may also provide opportunities to understand how to better design synthetic microbial communities for medicine, industry, and agriculture. Just as synthetic biologists rely on understanding regulatory networks and other interactions within cells to deconstruct and then reconstruct new metabolic pathways, synthetic microbial community ecologists will need to be able to first understand mechanisms underlying interactions within communities before piecing together synthetic microbial communities (Grosskopf and Soyer, 2014). Because MCoFFs have been designed—although in many cases unintentionally—for specific functions, can we use the pre-existing communities to teach us about design principles? Can we take microbes from disparate MCoFFs and combine them into new compositions not already found in food systems? What ecological or evolutionary constraints will prevent the construction of synthetic microbial communities, and can we use experimental community coevolution to overcome these constraints? Answering these questions with food microbial communities could lead to safer and more delicious foods while also developing much-needed principles of microbial community design.

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Cell

Leading Edge
Review

Meeting the Global Food Demand of the Future by Engineering Crop Photosynthesis and Yield Potential

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http://dx.doi.org/10.1016/j.cell.2015.03.019

Increase in demand for our primary foodstuffs is outstripping increase in yields, an expanding gap that indicates large potential food shortages by mid-century. This comes at a time when yield improvements are slowing or stagnating as the approaches of the Green Revolution reach their biological limits. Photosynthesis, which has been improved little in crops and falls far short of its biological limit, emerges as the key remaining route to increase the genetic yield potential of our major crops. Thus, there is a timely need to accelerate our understanding of the photosynthetic process in crops to allow informed and guided improvements via in-silico-assisted genetic engineering. Potential and emerging approaches to improving crop photosynthetic efficiency are discussed, and the new tools needed to realize these changes are presented.

An Emerging Yield Gap

Nothing is more important to human health and well-being than an adequate supply of food in terms of nutrition and calories. Although a significant proportion of the global population has suffered malnutrition over the last 50 years, it has been the result of failures in access to food, not in its global production. Indeed, over this period, we have seen surpluses of the major crops, which make shortages a very distant concern for most of the population. The most important primary foodstuffs, in terms of millions of metric tons (Mt) produced in 2013, were maize (1,018 Mt), paddy rice (746 Mt), wheat (713 Mt), and soybean (276 Mt) (Food and Agriculture Organization of the United Nations, 2015). These four crops account for about two thirds of calories consumed globally (Ray et al., 2013). Moreover, the average global yield per unit area of land (t/ha) for each of these crops has more than doubled since 1960, as illustrated for rice and wheat (Figure 1). So why bother worrying about food security now? One reason is that these global surpluses in staple crops have influenced the progressive decline in spending on plant science research and crop improvement, evident at the global level (Beintema and Elliott, 2009). However, this shift in funding may be myopic in the face of current global population and food consumption trends. Notably, the global population is expected to increase from just over 7 billion today to 9.5 billion by 2050, a 35% increase (USCB, 2015). An increasing proportion of the population will be urban, resulting in diets shifting increasingly from staples to processed foods, fortified with more meat and dairy products, which require large amounts of primary foodstuffs to produce. For example, 10 kg of feed is required to produce 1 kg live cattle (Smil, 2000). Thus, an increase in urban population will result in an increased demand for high-quality animal products, requiring an increase in crop production that is substantially faster than that estimated based solely on the projected population growth. This trend is expected to continue, and it is predicted that the world will need 85% more primary foodstuffs by 2050, relative to 2013 (Ray et al., 2013).

So is our current rate of increase in crop yields sufficient to meet this rising demand? It doesn't seem to be the case. If current rates of crop yield improvement per hectare are simply maintained into the future, supply will fall seriously below demand by 2050 (Figure 1; Ray et al., 2013). The resulting rise in global food prices may have the largest impact in the poorest tropical countries, which have the highest population increases. A compounding factor is that improvement in subsistence crops in these tropical countries is even slower than in our four leading crops. For example, the global average increase in yield per hectare of cassava, a major staple for sub-Saharan Africa, between 1960 and 2010 was 63%. This is less than half of the 171% increase for wheat over the same period (Figure 1). The problem is further compounded by the fact that the rate of improvement in yield of even our major crops in some areas of the globe is stagnating or even moving into reverse (Long, 2014; Long and Ort, 2010; Ray et al., 2012). Indeed, China, India, and Indonesia are the world's largest producers of rice, where yields per hectare across these countries increased by an average of 36% between 1970 and 1980 but only by 7% between 2000 and 2010 (Long, 2014). When faced with such numbers, one may rightfully ask: why are yield improvements stagnating?

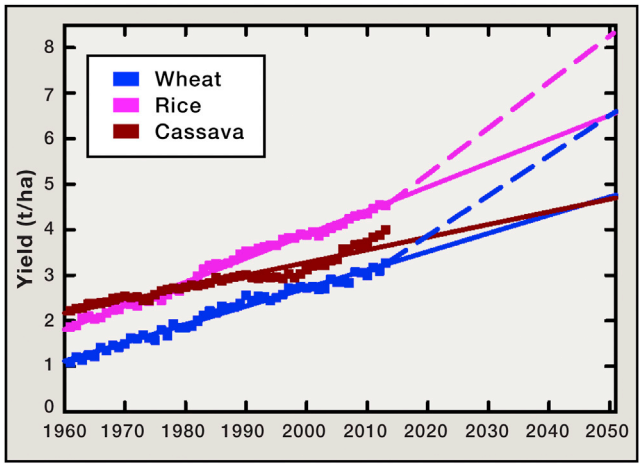

Figure 1. Annual Average Global Yields of Cassava, Rice, and Wheat from 1961 to 2013

Annual average yields for the entire globe in metric dry tons per hectare for each year from 1961 to 2013 for cassava, rice, and wheat (Food and Agriculture Organization of the United Nations, 2015). Solid lines are the least-square linear regressions fitted to these data and projected forward to 2050. The broken lines indicate the projected demand for rice and wheat, after Ray et al. (2013). The original data for cassava were provided as wet weight and are corrected here to dry weight, assuming a 70% water content.

Stagnation in Yield Improvement and Photosynthesis

The gains of the Green Revolution were achieved largely through improved genetics coupled with the enhanced agronomy and crop protection that allowed realization of the higher genetic yield potential. We can begin to understand these gains by defining them in mathematical terms. Yield potential (Y_p) is the mass of harvested material per hectare of land that a genotype of a crop can achieve in a given environment in the absence of biotic and abiotic stresses. Improved Y_p was achieved during the Green Revolution, in particular by selecting genotypes that partitioned more of their biomass into the harvested product. For example, the selection of dwarfed genotypes of wheat resulted in more biomass in the grain and less in the stem. This proportion of a plant's biomass that is invested into the harvested product, e.g., the grain of rice, is termed the partitioning efficiency or harvest index (ε_p). To a first approximation, the yield potential of a given genotype is then the product of the solar radiation received over the growing season by a unit area of land (Q) and the efficiencies with which the crop intercepts that radiation (ε_i), converts the intercepted radiation into biomass energy (ε_c), and then partitions the biomass into the harvested part of the plant (ε_p):

$$Yp = Q.\varepsilon_i.\varepsilon_c.\varepsilon_p..... \quad \text{(Equation 1)}$$

With reference to this equation, the Green Revolution increased ε_i and ε_p. In fact over the past 50 years, harvest index (ε_p) has almost doubled in the major grain crops and now stands at ~0.6 for modern cultivars of rice, wheat, and soy (Long et al., 2006b; Zhu et al., 2010). However, if these plants are to retain the structural components of the stems and ear or pod casings to support the seed at harvest, there is little prospect of further genetic improvement for this component of the equation. Similarly, interception efficiency (ε_i), that is the proportion of the visible sunlight that is intercepted by the crop over the growing season, has reached 0.8–0.9 for modern crop genotypes. Again, this suggests that this determinant of yield potential is also very close to its biological limits (Zhu et al., 2010). The one area in which there has been little or no improvement is in conversion efficiency (ε_c) of visible solar energy, which remains at about 0.02, and roughly one-fifth of the theoretical efficiency of 0.1 for C3 crops such as wheat and rice or 0.13 for C4 crops such as maize and sorghum (Zhu et al., 2008, 2010). Indeed, as it is clear that 50 years of conventional plant breeding has greatly improved ε_i and ε_p but not ε_c, this component of the equation appears to be a promising focus for further enhancement of yield potential.

Conversion efficiency depends on the efficiency of the process of photosynthesis, net of respiratory losses by the crop. Concern over global climate change motivated many studies of the effects of elevated CO_2 on crop production and photosynthesis. CO_2 is a limiting substrate for photosynthesis in C3 crops, so the primary effect is to artificially boost photosynthetic rate. Invariably, this results in increased yield (Ainsworth and Long, 2005; Kimball, 1983; Long et al., 2004, 2006a), demonstrating that there would be a clear benefit to yield if total crop photosynthesis could be increased genetically in crops (Long et al., 2006b). Yet, this also begets the question: if photosynthesis has such a strong influence on crop yield, why have traditional breeding and selection for higher yield delivered no or very small improvements in photosynthetic efficiency? There are several reasons for this effect. Within a crop species and its relatives, there is huge variation in ε_i and in factors affecting ε_p, such as the proportion of biomass invested in leaves during vegetative growth, rates of leaf growth, size of leaves, and leaf longevity. This has provided breeders with much variation in selecting for improved ε_i and ε_p. By contrast, the process of photosynthesis is highly conserved, not only within a crop species, but across a wide range of plants. Further, directed efforts have screened for germplasm with high light-saturated photosynthetic rates at the leaf level, and selection here has often been at the expense of other traits. For example, selection for higher light-saturated rates of leaf photosynthesis alone has often indirectly selected for lower total leaf area, offsetting any advantage at the crop level (Long et al., 2006b). This approach also ignores the fact that about half of crop carbon gain occurs under light-limited conditions (Long, 1993). How can we then approach increasing photosynthetic efficiency, and why might this be a timely strategy for a second Green Revolution when it was not for the first one?

Three factors make improving overall crop photosynthetic efficiency a possibility today. The first one is based on our understanding of the photosynthetic process. In the 50 years since the start of the first Green Revolution, knowledge of the photosynthetic process has exploded. From light capture by pigment molecules to production of storage carbohydrates; this fundamental process for all life on Earth is now understood in great detail. For higher plants, some algal species, and photosynthetic prokaryotes, not only is every step known, but the structures of the key proteins have been unraveled to high resolution to reveal the mechanism of their action, while the genes coding for the key components have been characterized. This includes

the recent isolation and characterization of the phycobilisome antenna complex and photosystems I and II from Synechocystis (Liu et al., 2013). This knowledge has facilitated the generation of kinetic models describing every discrete step of the entire processes of both C3 and C4 photosynthesis (Wang et al., 2014; Zhu et al., 2013). As a result, photosynthesis is undoubtedly the best known of all plant processes, and its similarity across all crops can be an advantage since what improves efficiency in one crop is likely to do so in another. The second factor lays in the emergence of high-performance computing (HPC). The rapid growth of computational power and new software tools has allowed the simulation of photosynthetic kinetic models of the complete process and application of optimization routines (Zhu et al., 2007, 2011). Not only can the metabolic pathways and their cellular organization be represented in silico, but there is now the opportunity to integrate them into realistic representations of the whole canopy of a crop, facilitating predictions of optimal distribution of resources at the sub-cellular, cellular, leaf, and whole-crop level (Drewry et al., 2014; Song et al., 2013; Tholen et al., 2012; Tholen and Zhu, 2011). HPC allows the in silico investigation of thousands of permutations of up- and downregulation of the genes and proteins involved in photosynthesis, or the impacts of the potential addition of foreign genes and pathways, to identify the best targets for practical manipulation (McGrath and Long, 2014; Xin et al., 2015). Finally, the third factor is the advance in genetic engineering. Genome editing and synthetic biology, once confined in the public domain to model species, is now becoming increasingly routine for a wide range of crops (Barampuram and Zhang, 2011). Combined, these three factors allow an informed and directed approach to engineering improved photosynthetic efficiency.

Does such a comprehensive strategy work? As an example, using this three-pronged approach, Zhu et al. (2007) predicted from applying evolutionary algorithms in silico that, for a given investment of resources into photosynthetic carbon metabolism, there should be significant re-allocation of resources between the proteins involved to maximize efficiency. This was predicted to deliver a 60% increase in photosynthetic efficiency, and the largest single change was an increase in investment in the enzyme sedoheptulose-1:7-bisphosphatase (SBPase). The benefit of upregulation of this enzyme was also predicted to increase as atmospheric CO_2 levels rise (Zhu et al., 2007). Subsequent upregulation of this enzyme in tobacco was shown to substantially increase the productivity of a field crop of tobacco, and this increase in photosynthesis was greater under an open-air elevation of CO_2 in the field (Rosenthal et al., 2011).

If such gains can be achieved, then why has natural evolution not already optimized the system? First, evolution in the wild selects for survival and fecundity and not directly for productivity. Second, the carbon dioxide concentration of the atmosphere averaged over the past 25 million years—and in which the ancestors of our crop plants evolved—was about 220 μmol mol^{-1} (Zhu et al., 2004b). Today, it is almost double that concentration, and most of that increase has occurred in the last 100 years, which is a too-short period of time for our crops to become adapted. Therefore, our challenge is to identify new targets and develop strategies to achieve these predicted gains.

Targets for Increasing Crop Photosynthetic Efficiency

From our fundamental understanding of photosynthesis, what are the likely targets for systems and synthetic improvement of efficiency in crops? As noted above, the achieved net photosynthetic efficiency of our crops falls far short of the theoretical; so what are the weak links in the process? Photosynthesis can be divided into two stages, sometimes referred to as the light and dark reactions. The light reactions concern the capture of light energy by chlorophyll and associated pigments, water splitting, and electron transport on the chloroplast membrane reducing NADP and providing the proton gradient that powers phosphorylation of ADP. In the dark reactions, the resulting NAPDH and ATP power the Calvin cycle, which assimilates carbon dioxide and reduces it to carbohydrate. Examination of the steps involved in this process shows that a minimum of eight photons are required for the assimilation of one molecule of CO_2 and release of one molecule of O_2 from water splitting (Blankenship et al., 2011). Analyses of the actual photon requirement of leaves of a wide range of plants and crops and of young and old leaves show that, under low light and in the absence of other stresses, the photosynthetic apparatus of most leaves comes very close to the theoretical requirement of eight photons (Björkman and Demmig, 1987; Long et al., 1993). This shows that the primary processes can already operate close to maximum efficiency. However, as absorbed light is increased, the efficiency of photon use declines. This is because of limitation in either electron transport or capacity to utilize the ATP and NADPH produced. There are nevertheless two apparent ways in which the efficiency of light capture and energy transduction could still be increased for field crops; the first one is by engineering pigments that could utilize more of the sunlight's spectrum, and the second one is by overcoming light saturation of the downstream photosynthetic processes. First, the pigment systems of plants, like the green algae from which they evolved, can only effectively use the visible spectrum, with a very small extension into the near infra-red and UV-A spectra. This means that more than half of solar energy is unavailable (Zhu et al., 2008). Other algae and some photosynthetic bacteria use pigments that are able to capture and utilize longer wavelengths of near infra-red radiation. Re-engineering the photosystems and their collection antennae that drive electron transport could raise the maximum efficiency by allowing use of another ~20% of the available solar energy (Blankenship et al., 2011). This would be particularly valuable in the lower levels of crop leaf canopies in which carbon assimilation will rise linearly with increased efficiency of light capture (Long, 1993). Another approach suggested as beneficial from modeling is to reduce the antenna size of the photosystems in upper canopy leaves. The antennae are the chlorophyll molecules that capture light energy and feed it to the photosystem centers (PS1 and PSII) that drive electron transport, delivering the NADPH and ATP that power carbon dioxide assimilation. Modeling suggests that these antennae are too large, trapping more light energy than they may use. This may have an evolutionary origin because, in the wild, an individual that can trap more light in its upper leaves denies light to competing plants underneath, even if it cannot itself use the light. But, in a crop monoculture, it is disadvantageous, and reducing antenna size could save resources and allow more light to reach lower leaves (Ort

et al., 2011). Decreasing the antenna size will also decrease loss of absorbed light energy in the form of heat and fluorescence for leaves under both high and low light levels (Zhu et al., 2005; Blankenship and Chen, 2013). Chlorophyll-a-oxidase has been reported to be related to antenna size (Masuda et al., 2003) and can thus be a target for manipulation to increase light capture efficiency and assimilation.

Downstream limitations of assimilation also exist, where in high light, i.e., between full and approximately one-third of full sunlight (i.e., 550–2,200 μmol m^{-2} s^{-1} visible photons), the photosynthetic apparatus is capturing more light energy than it may utilize and is saturated. If chlorophyll molecules remain in an excited state, the excitation energy can be transferred to oxygen, producing a range of oxidizing radicals. These in turn can destroy the photosynthetic apparatus (Aro et al., 1993; Long et al., 1994). Plants protect themselves against excess radiation by changes within the apparatus induced through the de-epoxidation of the xanthophyll pigment violaxanthin to zeaxanthin. These and associated changes result in dissipation of absorbed excess energy harmlessly as heat (Ahn et al., 2008; Müller et al., 2001; Niyogi, 1999). However, in a crop canopy, photosynthetic cells can pass rapidly from high light to low light as a cloud passes the sun or as the continuous change in solar angle can abruptly place a cell in one leaf in the shade of another leaf. Suddenly then, a photosynthetic cell is transferred from light saturation to light limitation, and here dissipation of absorbed light energy as heat will lower the efficiency of photosynthesis. Modeling of the dynamics of these light fluctuations shows that this could cost up to 30% of potential assimilation (Zhu et al., 2004a). Again, there are algal systems that can relax this heat dissipation far more rapidly, offering synthetic opportunities to overcome this loss. Improved understanding of the mechanism from model plants such as *Arabidopsis* indicates additional systems opportunities to enhance this recovery of photosystem II (PSII) efficiency. This is through up- regulation of genes coding for enzymes involved in inter-conversion of intermediates of the xanthophyll cycle and the interaction of these intermediates with the PSII complex (Murchie and Niyogi, 2011).

What are the limitations in the "dark" reactions of photosynthesis? Application of a widely validated steady-state biochemical model of photosynthesis (Farquhar et al., 1980, 2001) to crop leaves shows that, at light saturation, the process in vivo is typically co-limited by capacity for carboxylation and capacity for regeneration of the acceptor molecule for carboxylation, ribulose-1:5 bisphosphate (RubP) (Long et al., 2004). Capacity for carboxylation in C3 crops is determined by the activity of a single enzyme, RubP carboxylase/oxgenase (Rubisco). Rubisco typically represents 50% of the soluble protein in the leaf and is the most abundant protein on the planet (Spreitzer and Salvucci, 2002). Why is such an abundant enzyme limiting? Rubisco can catalyze both the carboxylation and oxygenation of RubP. If RubP is oxygenated, then a two-carbon compound, phosphoglycolate, is formed. Plants metabolize this product through a complex pathway involving peroxisomes and mitochondria to regenerate phospho-glycerate (PGA; Figure 2). PGA is a C3 intermediate of the Calvin cycle, but it is produced here at the cost of the loss of a molecule of CO_2 and the use of a significant amount of reductive and phosphorylating capacity generated by the light reactions (Figure 2) (Farquhar and Caemmerer, 1982). This process of oxygen consumption and CO_2 release is termed photorespiration. Photorespiration imposes a large penalty on net photosynthetic efficiency, which increases with temperature. This is because the specificity of Rubisco for CO_2 declines with temperature, so loss due to photorespiration rises from ~30% in cool climates to more than 50% in hot climates (Long et al., 2006b). To combat photorespiration, it appears that Rubisco in plants has evolved to become more specific for CO_2, but achieving specificity in evolution appears to have been at the expense of speed of catalysis. Indeed, the catalytic rate of Rubisco from plants is one of the slowest of any enzyme-catalyzed reactions at ~3.7 per active site per second (Parry et al., 2013; Tcherkez, 2013; Zhu et al., 2004b). Modern forms of Rubisco therefore represent a compromise between specificity (τ) and catalytic rate (k_{cat}). However, this compromise appears optimized for the past atmospheric CO_2 concentration of about 220 μmol mol^{-1} and not today's concentration of 400 μmol mol^{-1} (Zhu et al., 2004a). Computer simulation of crop canopies suggests that engineering a Rubisco optimized to today's atmosphere requiring a higher kcat, even at the expense of a lower specificity, could increase photosynthetic carbon gain by a crop canopy by up to 30% for the same total amount of enzyme (Zhu et al., 2004a).

Carbon dioxide is a competitive inhibitor of the oxygenation reaction of Rubisco. Evolution has exploited this in some photosynthetic organisms by the addition of structures to compartmentalize Rubisco and pathways that concentrate CO_2 in that compartment. C4 photosynthesis is one solution that has evolved independently over 60 times (Sage et al., 2012). In C4 plants, which include the crops maize, sorghum, sugarcane, and grain amaranth, Rubisco is isolated to an inner green bundle sheath surrounding the leaf veins. In these plants, carbon dioxide is first captured by carboxylation of phosphoenolpyruvate (PEP) to form a C4 dicarboxylate in an outer photosynthetic tissue or mesophyll and is then transferred to the inner tissue that it surrounds, the bundle sheath. Here, it is decarboxylated, releasing pyruvate that is then recycled back to the outer tissue, where it is phosphorylated back to PEP to complete the cycle (Long and Spence, 2013). In essence, this C4 cycle serves as a light-energy-driven CO_2 concentrating mechanism, which largely eliminates photorespiration (Sage et al., 2012; von Caemmerer and Furbank, 2003). The additional energy required by the C4 cycle is, in most circumstances, less than would be lost in photorespiratory metabolism (Long and Spence, 2013). C4 plants generally have higher rates of photosynthesis and include the most productive crops and plants known (DeLucia et al., 2014; Long and Spence, 2013; Piedade et al., 1991). Indeed, one approach to increasing photosynthetic efficiency in C3 crops such as wheat and rice is to convert them to C4 plants. A major effort is underway to achieve this in rice; however, it requires many changes in both anatomy and expression of Calvin cycle enzymes, as well as inserting and expressing the genes of C4 photosynthesis (von Caemmerer et al., 2012). Nevertheless, the fact that this process has successfully and independently evolved over 60 times in nature suggests that this is achievable, although it will require further understanding of the genetic basis of the dimorphic photosynthetic tissue and localization of components of the C4 and Calvin cycles.

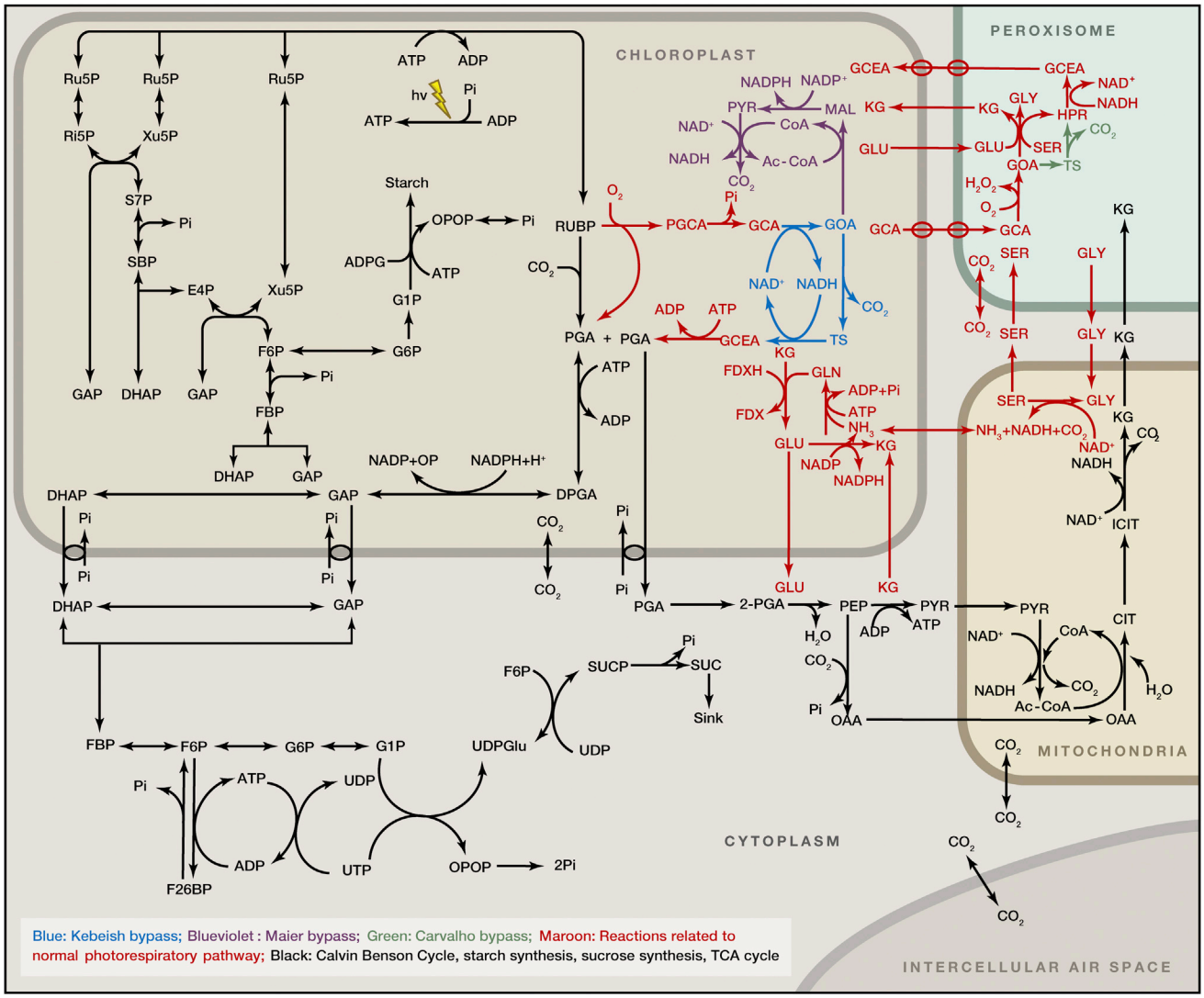

Figure 2. Schematic Representation of Photosynthetic C3 Metabolism, with Both the Native and Potential Synthetic Pathways of Photorespiratory C2 Metabolism

The C3 photosynthetic or Calvin cycle and pathways to immediate carbohydrate products in the cytosol are indicated in black. Pathways for C2 metabolism are indicated as follows: maroon, the native photorespiratory C2 pathway; blue, the synthetic bypass described by Kebeish et al. (2007); violet, the bypass described by Maier et al. (2012); and green, the bypass described by Carvalho et al. (2011) in green. Abbreviations: ADPG, ADP-glucose; AcCoA, Acetyl- Coenzyme A; CIT, Citric acid; CoA, Coenzyme A; DHAP, Dihydroxyacetone-phosphate; DPGA, 1,3- bisphosphoglycerate; E4P, Erythrose 4-phosphate; F6P, Fructose 6-phosphate; FBP, Fructose 1,6- bisphosphate; FDXH, Reduced ferrodoxin; FDX, Oxidized ferrodoxin; F26BP, Fructose 2,6-bisphosphate; G1P, Glucose 1-phosphate; G6P, Glucose 6-phosphate; GAP, Glyceraldehyde 3-phosphate; GCA, Glycollate; GCEA, Glycerate; GLU, Glutamate; GLN, Glutamine; GLY, Glycine; GOA, Glyoxylate; HPR, Hydroxypyruvate; ICIT, Isocitric acid; KG, a-Ketoglutarate; MAL, Malate; OAA, Oxaloacetic acid; OPOP, Pyrophosphate; PGA, 3-Phosphoglycerate; 2-PGA, 3-Phosphoglycerate; PGCA, 3-Phosphoglycollate; PYR, Pyruvate; Ri5P, Ribose 5-phosphate; Ru5P, Ribulose 5-phosphate; RuBP, Ribulose 1,5-biphosphate; S7P, Sedoheptulose 7-phosphate; SBP, Sedoheptulose 1,7- bisphosphate; SER, Serine; TS, tartronic semialdehyde; T3P, Triose phosphate; UDPGlu, Uridine Diphosphate Glucose; SUC, Sucrose; SUCP, Sucrose phosphate; UDP, Uridine 5′-diphosphate; UTP, Uridine-5′-triphosphate; Xu5P, Xylulose 5-phosphate; and Pi, phosphate. This image is redrawn and adapted from Xin et al. (2015).

Cyanobacteria, the ancestors of modern day crop chloroplasts, use a different method of concentrating CO_2 at Rubisco. These prokaryotes actively uptake bicarbonate into their cells. Within the cells, both Rubisco and carbonic anhydrase are localized within icosahedral protein shell bodies termed carboxysomes. Here, carbonic anhydrase catalyzes the formation of CO_2, serving to concentrate CO_2 around Rubisco to a sufficient level to minimize oxygenation and photorespiration (Badger and Price, 2003; Price, 2011). This appears simpler than converting a C3 plant to a C4 because it does not require the creation of two distinct photosynthetic tissues but instead requires the addition of bicarbonate and carbon dioxide pumps to the chloroplast membrane and production of carboxysomes within the chloroplast. A diffusion-reaction model of this system suggests that addition of these basic components would increase photosynthesis as much as 60%, whereas addition of the bicarbonate

pumps alone would increase photosynthesis in C3 leaves by ~9% (McGrath and Long, 2014). Ideally these components would be coded for and synthesized within the chloroplast by transformation of the remnants of the cyanobacterial DNA that persists in modern-day chloroplasts (Martin et al., 2002). However, transformation of chloroplast DNA has so far only succeeded in a few plants, notably tobacco and potato, but as yet not in any cereal (Scharff and Bock, 2014). An alternative may be to code for these components by nuclear transformation with transit peptides and membrane transporters. Many eukaryotic algae also include inorganic carbon concentrating mechanisms to suppress the oxygenase activity of Rubisco and photorespiration (Meyer and Griffiths, 2013). These also require bicarbonate transporters in the cell and chloroplast membrane. Within the chloroplasts of these algae, Rubisco concentrates in a region, typically surrounded by starch, and is termed the pyrenoid (Giordano et al., 2005). Pyrenoids appear to function similarly to carboxysomes, although the dynamic nature of their structure and genetics are less well understood, despite important recent advances (Meyer and Griffiths, 2013; Mitchell et al., 2014; Engel et al., 2015). So although transferring a eukaryotic-concentrating mechanism to other eukaryotes may appear more tractable, more genetic information is needed to understand how this might be engineered. Rubisco is not the only carboxylase in nature, and there are at least five known pathways of carbohydrate synthesis from CO_2 that use other carboxylases (Fuchs, 2011). However, only one of these, the 3-hydroxypropionate (3-HPA) bicycle, appears oxygen insensitive (Mattozzi et al., 2013). These existing pathways or totally synthetic pathways could be introduced into higher plants (Bar-Even et al., 2010). A challenge here, though, is that the intermediates of the Calvin cycle are integrally linked to much of essential plant metabolism. Introduction of such a different pathway would have complex effects on the whole of plant metabolism and may require successful re-engineering far beyond carboxylation.

A further opportunity to address the cost of photorespiration is to engineer a more efficient pathway for metabolism of the first product of the oxygenase reaction, phosphoglycolate. Plants and green algae use a single energy-consuming and protracted pathway involving the chloroplast, peroxisome, and mitochondrion, with the release of both carbon dioxide and ammonia in order to recover PGA that is then re-assimilated into the Calvin cycle. This is shown by the red intermediates in Figure 2. Prokaryotes have at least three simpler pathways for metabolism of phosphoglycolate to PGA (Carvalho et al., 2011; Maier et al., 2012; Maurino and Peterhansel, 2010; Xin et al., 2015). One that involves just three enzyme-catalyzed steps has been engineered into the chloroplast of *Arabidopsis* with an improvement in net photosynthetic efficiency (Kebeish et al., 2007). The three pathways and how they could be engineered into crop photosynthetic carbon metabolism are illustrated in Figure 2. A simulated energy balance analysis of the complete photosynthetic system with addition of each of these pathways has, in fact, predicted that this three-step pathway is the only one that would actually increase net photosynthetic efficiency (Xin et al., 2015), demonstrating the power of in silico analysis in directing practical manipulations.

As noted above, metabolic control of CO_2 assimilation is commonly shared between Rubisco and capacity for regeneration of RubP. When account is taken of the Calvin cycle, electron transport, photorespiratory metabolism, and transfer of intermediates of the Calvin cycle to storage and transport carbohydrates, starch, and sucrose, more than 60 reactions are involved. Representation of this system in silico and application of an evolutionary algorithm has indicated several pressure points, including SBPase, which, if upregulated, could increase photosynthesis by 60% without additional resources (Zhu et al., 2007). Further gains may also be achieved by altering the arrangement, amount, and color of leaves in a crop canopy (Drewry et al., 2014; Long et al., 2006b; Zhu et al., 2010). In full sunlight, the uppermost leaves of most crop canopies capture most of the incoming sunlight, which is more than they can use, whereas the lower leaves receive far less light than they could utilize; likely an evolutionary hold over. The wild ancestors of our crop plants were subject to selection as individuals growing in a competitive environment. By capturing most light on their upper leaves, even when this could not be effectively used, competitors growing below were denied this light (Zhu et al., 2010). But in a monoculture of genetically identical crop plants, this strategy is disadvantageous. By making upper leaves more vertical and lighter in color, light can be more evenly distributed, allowing up to a 60% increase in carbon gain by a canopy of the same total leaf area per unit ground area, while achieving improvements in water use efficiency (Drewry et al., 2014; Zhu et al., 2010). Finally, respiration is the other component determining the net efficiency with which crops convert intercepted solar radiation into biomass. Far less is known about plant respiration, in particular, whether it can be decreased without impacting growth and maintenance processes (Costa et al., 2014; Logan, 2007; Millar et al., 2011; Peckmann et al., 2012; Sweetlove et al., 2010). Early genetic work with ryegrass suggested that lines with lower respiration did have higher productivity (Robson, 1982), although this has not subsequently been confirmed in other crops. However, it clearly represents an area in need of far more investment.

Table 1 summarizes the major possible methods of improvement of photosynthetic efficiency that are currently apparent, the likely timescale, and the potential gain. Although some are clearly more tractable at the present time than others, we have insufficient knowledge to favor one approach over another. Indeed, the problem represented by Figure 1 is large enough to suggest that we should be actively and urgently pursuing all of these approaches, and although the potential improvements are not necessarily additive, they are not antagonistic.

Facilitating Translation of Research Opportunities to Crops

Some of the most tractable methods to improve photosynthetic efficiency include systems/synthetic biology, genetic engineering, and computational modeling strategies as part of a new Green Revolution. Unicellular green algae such as *Chlorella* and *Chlamydomonas*, as well as model plants with short life cycles, rapid transformation systems, and deep functional knowledge of their genomes such as *Arabidopsis*, remain crucial tools for tests of concept for a number of these potential

Table 1. This Table Lists the Manipulations That Could Be Undertaken to Improve Photosynthetic Efficiency in C3 Crops, the Type of Manipulation, and the Model Estimated Improvement in Efficiency of Conversion of Received Light Energy into Crop Biomass Relative to Today's Best Cultivars

	Manipulation	Type	Efficiency Gain	Timescale	Additional Benefits
1	extend usable spectrum of crop photosynthesis into NIR	CSyn	10%–30%[a]	L	could be used to power 3 or improve value of 10. C4
2	more rapid relaxation of heat dissipation at PSII	Syn	30%[i]	S	synergistic with all other changes. C4
3	convert C3 crops to C4	Syn	30%[c,e]	L	improved WUE and NUE
4	add cyanobacterial or microalgal CO2/HCO3 pumps	Syn	5%–10%[d]	M	improved WUE and NUE
5	add cyanobacterial carboxysome system	CSyn	60%[d]	L	improved WUE and NUE
6	add algal pyrenoid CO2 concentrating system	CSyn	60%[d]	L	improved WUE and NUE
7	substitute forms of Rubisco better adapted to today's CO_2	CSyn, B	15%–30%[h,j]	L	improved WUE and NUE
8	synthetic photorespiratory bypasses	Syn	15%[c,f]	S	improved WUE and NUE
9	optimize regeneration of RubP	Sys, B	60%[g]	S	synergistic with all; improved NUE. C4
10	transmit more light to lower canopy leaves	B, Syn	15%–60%[b,c]	S	synergistic with 1, and 3 thru 9. improved WUE and albedo. C4

CSyn indicates synthetic addition of foreign genes to the chloroplast or plastid genome; Syn indicates synthetic addition to the nuclear genome; Sys indicated up- or down-regulation of existing genes; and B indicates that the improvement may be tractable by breeding given adequate molecular markers for the specific genes. The efficiency gains are from modeled estimates and are largely untested; these vary greatly depending on different assumptions and can vary with environmental conditions. For example, the benefits of items three through eight will increase with temperature and so give the greatest increases in warm climates. Timescale is an estimated time to obtain material that could be used in a breeding program. S represents a 1–5 year timescale, since these have already been demonstrated in model plant species or actual crops as providing some clear improvement; M indicates a 5–10 year timescale; and L indicates a 10–30 year time scale. These involve manipulations that require as-yet-unachieved goals, such as plastid transformation, or a full understanding of what makes a plant C4. It should be noted that, with adequate resource, there is no reason to believe that these goals cannot be achieved. All timescales are estimates assuming adequate investment for intensive effort and the needed resources. Additional benefits indicate synergies, i.e., where 1+1 > 2, and simultaneous improvements in either the efficiency of water use (WUE) or of nitrogen use (NUE) per unit of biomass are improved. C4 indicates that this manipulation would also improve efficiency of carbon gain in C4 crops, such as maize and sorghum. Sources.

[a]Blankenship et al. (2011).
[b]Drewry et al. (2014).
[c]Long et al. (2006a).
[d]McGrath and Long (2014).
[e]von Caemmerer et al. (2012).
[f]Xin et al. (2015).
[g]Zhu et al. (2007).
[h]Zhu and Long (2009).
[i]Zhu et al. (2004a).
[j]Zhu et al. (2004b).

improvements (Table 1). However, these cannot predict or represent the complexities of a closed-crop canopy and its photosynthetic performance in the field. Transformation of our major crops, on the other hand, is generally slow and has been limited to a few public laboratories. Solanaceous crops have proved some of the easiest to transform. Tobacco is a valuable test bed, it is easily transformed, and it forms a closed canopy in the field with many of the same characteristics of food crops (Figure 3). Tobacco can serve as a key test bed to identify the most promising manipulations that might then be moved on to the more difficult tasks of transforming crops such as wheat, rice, soy, or cassava. However, conventional transformation of plants with constructs to up- or down-regulate specific genes or to introduce synthetic pathways through agro-bacterium or biolistic addition results in near-random insertions. This presents a challenge when quantifying and testing the improvement made by a specific addition to crop carbon gain in the field. Positional effects mean that no one event is the same, and some may be lethal in homozygotes of the second generation (T1) in which they have knocked out key genes. This necessitates the testing of many events as replicated stands of the transformants in the field, limiting the number of constructs that might be tested (Figure 2). Tools that would allow insertion of constructs at the same point in the genome, a point that does not interfere with expression of other genes, would decrease variability between events and increase comparability of transformations with different constructs. Recombination-mediated genetic engineering (recombineering), Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and, in particular, clustered, regularly interspaced, short palindromic repeats (CRISPRs) widely used in engineering of microbial genomes provide a means to achieve directed insertions in crops (Jiang et al., 2013; Shan et al., 2013). Testing would be further facilitated by the development of haploid plants for transformation, so that homozygotes can be obtained rapidly (Lee et al., 2014; Suelter et al., 2014). Although, in the evolution of

Figure 3. The Pipeline of Transformation of Leaf Discs of Tobacco with Constructs for Improved Photosynthetic Efficiency through Regeneration on Selective Media, Growth of the Initial Transformants to Seed, and Then Testing of Transgenes in Replicated Field Plots

chloroplasts, most genetic information of the ancestral cyanobacteria has been transferred to the nucleus, the vestigial DNA codes for some key proteins of the photosynthetic apparatus, including the larger part of Rubisco. Achieving a number of the potential improvements suggested here requires or would benefit from successful transformation of the chloroplast DNA. As noted earlier, this has only been achieved in a few species and, as yet, in none of the major crops (Scharff and Bock, 2014). But there appears to be no fundamental barrier to achieving this, except adequate investment.

Sufficient knowledge is now available that modeling a whole crop plant in silico might be achievable (Chew et al., 2014), thus creating a tool that would be both a framework for testing hypotheses on improving net carbon gain and production and for applying optimization routines to improve efficiency (Figure 4). Mechanistic models of gene expression networks, proteins, metabolic pathways, shoot and root development, and canopy microclimate have all been developed. Although these models have been used in isolation to predict synthetic and systems means to improve photosynthetic efficiency, they ignore interactions with the rest of the plant system or crop ecosystem. Multi-scale modeling that integrates these different models can aid in system-wide predictions of photosynthetic efficiency across scales (Figure 4).

Why Now and Not 2050?

This article has focused on genetic yield potential improvement to increase yield per hectare as a means to protect against a potential future shortage of primary foodstuffs (Figure 1). There are of course other means of increasing food supply by using more land and by raising yields on all farms to those achieved by the best farmers. However, our major food crops require good soils and water supply to achieve high yields, and there is little land of this quality that is not already in production. Indeed urban sprawl, desertification, salination, and exhaustion of aquifers that have been used for irrigation suggest that less, not more, land may be available into the future (Alexandratos and Bruinsma, 2012). Crops could be raised on poorer land than that used today but with lower yields and greater risk of environmental and biodiversity degradation. Yields for a given crop can vary greatly between farms and countries. For instance, the average yield of maize in the USA in 2013 was 10.0 t/ha and in Zimbabwe 0.9 t/ha, even though both have similarly good climatic conditions for raising the crop (Food and Agriculture Organization of the United Nations, 2015). Raising yields to those achieved by the best farmers is an important aim but depends greatly on internal policies and farmer access to advice, seed, and fertilizers, none of which can have certainty into the future. So while in the best of worlds, seed with increased yield potential might not be necessary, we cannot and should not take that risk. Given the 20 to 30 year gap

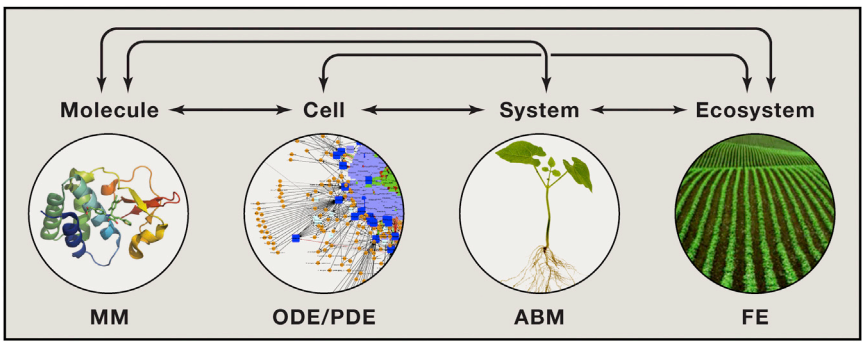

Figure 4. Multi-scale Modeling Concept
Models at different levels, generated with different mathematical strategies, inform one another and bridge the critical gaps in our knowledge of fundamental plant behavior. Examples of model types include MM, molecular modeling; ODE, ordinary differential equations; PDE, partial differential equations; ABM, agent based modeling; and FE, finite element modeling.

between demonstration of innovative solutions at the experimental level and provision of seed to farmers, the need to bridge and accelerate the gap between molecular engineering and practical crop breeding to achieve higher yields cannot be postponed, especially considering the forecast situation for 2050.

ACKNOWLEDGMENTS

S.P.L and X.-G.Z. acknowledge the support of the Bill & Melinda Gates Foundation RIPE Project, the US Department of Energy ARPA-E PETRO Program, and the Chinese Academy of Sciences. S.P.L. also acknowledges support from the National Science Foundation (grant number EF-1105584). We thank Ms. Haley Ahlers and Dr. Diwakar Shukla of the University of Illinois for help in the preparation of Figures 3 and 4, respectively.

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Cell

Nutrient-Sensing Mechanisms across Evolution

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http://dx.doi.org/10.1016/j.cell.2015.02.041

For organisms to coordinate their growth and development with nutrient availability, they must be able to sense nutrient levels in their environment. Here, we review select nutrient-sensing mechanisms in a few diverse organisms. We discuss how these mechanisms reflect the nutrient requirements of specific species and how they have adapted to the emergence of multicellularity in eukaryotes.

Introduction

All organisms have the capacity to sense the presence and absence of the nutrients required to generate energy and the building blocks of cells. In this Review, we survey a variety of nutrient-sensing strategies and discuss how these mechanisms have evolved to suit the particular needs and environments of diverse organisms.

We first illustrate how varied sensing mechanisms can be using examples from unicellular organisms, including the sensing of amino acids by *E. coli* and of ammonium and glucose by *S. cerevisiae*. We then shift our focus to sensing pathways conserved in most eukaryotes, including those anchored by the AMPK, GCN2, and TOR kinases (Figure 1). We emphasize how, in multicellular organisms, the architectures of these core pathways has been adapted to respond to hormones in addition to nutrients and to control feeding. Furthermore, we highlight how the emergence of subcellular compartments in eukaryotes allows for new ways to store and sense nutrients.

Prokaryotes

Bacteria have evolved many interesting mechanisms for sensing diverse nutrients, undoubtedly an adaptation to living in environments where the concentrations and types of nutrients can vary unpredictably. We have chosen to discuss mechanisms that serve as examples of important concepts, such as the use of enzymes or receptors to detect molecules of interest or the indirect sensing of a nutrient through the levels of metabolites generated from it. The variety of post-translational modifications that bacterial-sensing pathways use, from phosphorylation to adenylylation and methylation, is remarkable, as is the concentration range over which some nutrients can be detected.

Chemoreceptors: Coupling Extracellular Nutrient Concentrations to Cell Motility

Bacteria can face large fluctuations in the levels of nutrients in their environment, and so motile species couple nutrient sensing to taxis to bias their movements toward higher nutrient concentrations. While we focus on nutrient-regulated chemotaxis in *E. coli*, the process is conserved in many prokaryotes (Szurmant and Ordal, 2004).

E. coli swim by rotating their flagella, bundles of filaments localized at the pole and powered by a rotary motor (Berg, 2008; Eisenbach, 1996). The motor is bidirectional: counterclockwise rotation produces smooth swimming, whereas clockwise rotation leads to random tumbling due to dispersal of the flagellar filaments (Larsen et al., 1974; Turner et al., 2000). The default rotation of the motor is counterclockwise, and nutrients signal through transmembrane chemoreceptors to maintain this state to promote directed movement along a nutrient gradient. The absence of nutrients triggers a pathway that permits flagella to alternate between clockwise and counterclockwise rotations so that cells forage their environment in a random walk (Berg and Brown, 1972; Sourjik and Wingreen, 2012).

E. coli express five dimeric, single-pass transmembrane chemoreceptors—Tar, Tsr, Tap, Trg, and Aer—which function as distinct nutrient sensors. In aggregate, they allow *E. coli* to detect and respond to a broad spectrum of extracellular molecules, with aspartate, maltose, Co^{2+}, and Ni^{2+} binding to Tar (Reader et al., 1979; Wang and Koshland, 1980); ribose and galactose to Trg (Kondoh et al., 1979); flavin adenine dinucleotide to Aer (Szurmant and Ordal, 2004); serine to Tsr; and dipeptides to Tap (Hedblom and Adler, 1980; Manson et al., 1986). Of the five receptors, Tar and Tsr are the most abundant (Sourjik and Berg, 2004). Remarkably, the chemoreceptors sense ligand concentrations as low as 3 nM and function over a concentration range of five orders of magnitude (Mesibov et al., 1973). This high sensitivity stems from the clustering at the cell pole of the receptors into higher-order arrays, enabling one ligand-binding event to affect multiple neighboring receptors and effectors (Bray et al., 1998; Briegel et al., 2009; Kentner et al., 2006; Levit et al., 2002; Maddock and Shapiro, 1993; Sourjik and Berg, 2004; Zhang et al., 2007), which presumably allows cells to detect even highly dilute nutrient environments.

The chemoreceptors signal through CheA, a homodimeric histidine kinase that constitutively associates with the chemoreceptors and its adaptor protein, CheW (Figure 2A). In the absence of a ligand, CheA phosphorylates the associated response regulator, CheY (Borkovich et al., 1989; Hess et al., 1987, 1988; Stock et al., 1988), which then diffuses to the

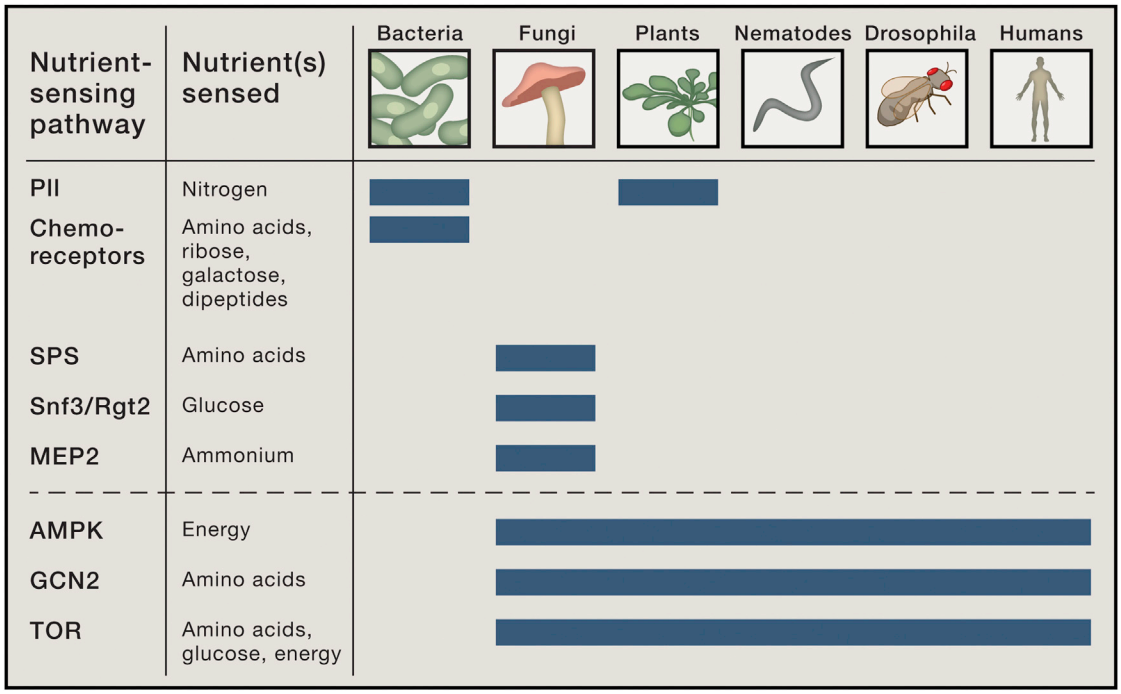

Figure 1. Nutrient-Sensing Pathways throughout Evolution
An overview of the nutrient-sensing pathways described in this Review. Pathways specific to unicellular organisms are denoted, followed by the sensing pathways that are conserved from yeast to man. Blue bars indicate the presence of the pathway within the denoted species or organism.

flagellar motor to promote clockwise rotation (Dyer et al., 2009; Sarkar et al., 2010; Scharf et al., 1998; Welch et al., 1993). Nutrients diffuse into the periplasm through channels in the outer membrane and directly or indirectly contact chemoreceptors to trigger a conformational change that inhibits CheA and CheY (Ottemann et al., 1999), causing bacteria to swim in a positive direction for longer periods of time.

A crucial aspect of chemotaxis is adaptation—the ability to restore prestimulus behavior. Chemoreceptor methylation by the methyltransferase CheR and demethylation by the methylesterase CheB facilitates adaptation (Anand and Stock, 2002; Borkovich and Simon, 1990; Bren and Eisenbach, 2000; Kondoh et al., 1979). In addition to phosphorylating CheY, the CheA kinase phosphorylates and activates CheB, which demethylates the chemoreceptor and reduces its capacity to activate CheA despite persistently low ligand concentrations. Conversely, sustained increases in nutrient concentrations lead to an accumulation of receptor methylation over time, as CheB is inactive and CheR constitutively active. This enhances the ability of the chemoreceptor to stimulate CheA despite constant high concentrations of attractant. Thus, CheB promotes adaptation to decreasing levels of attractants while CheR promotes adaptation to increasing levels of attractants. Methylation therefore resets the signaling state of the receptors so that *E. coli* can adapt to the present environment and be poised to respond to subsequent changes (Wadhams and Armitage, 2004; Weis and Koshland, 1988).

The capacity to couple motility to nutrient concentrations allows bacteria to forage for limited resources. However, all bacteria, motile or not, must possess mechanisms that relay nutrient levels to the metabolic systems that counteract nutrient deficits so as to maintain the metabolites needed for viability and growth. One such mechanism is the PII protein pathway, which uses a cascade of post-translational modifications to control nitrogen assimilation.

PII Proteins: Controllers of Nitrogen Assimilation

Under nitrogen-limiting conditions, many prokaryotes increase nitrogen assimilation by synthesizing nitrogen-containing organic molecules, such as amino acids, from inorganic nitrogen compounds in the environment. Assimilation occurs via a dedicated glutamine synthetase (GS)/glutamate synthase (GOGAT) cascade that generates glutamate from 2-oxoglutarate (2-OG; also known as α-ketoglutarate), ammonium, and ATP. The first step of the process, catalyzed by GS, produces glutamine, and thus its presence represents nitrogen sufficiency, while 2-OG, its precursor, signals nitrogen deficiency (Forchhammer, 2007; Leigh and Dodsworth, 2007). The PII proteins play a key role in sensing nitrogen deficiency, and although they vary greatly in structure and function, they are found in most prokaryotes and plants (Heinrich et al., 2004) (Figure 1).

Within the PII superfamily of proteins the related GlnB and GlnK proteins in *E. coli* have been particularly well studied (Leigh and Dodsworth, 2007). These proteins have significant homology only to other PII proteins and form homotrimeric complexes. Their main function is to control the adenylylation of GS, which inhibits it and thus reduces nitrogen assimilation (Figure 2B) (Leigh and Dodsworth, 2007). The PII proteins exist in two forms: unmodified and uridylylated. Only when unmodified do they bind

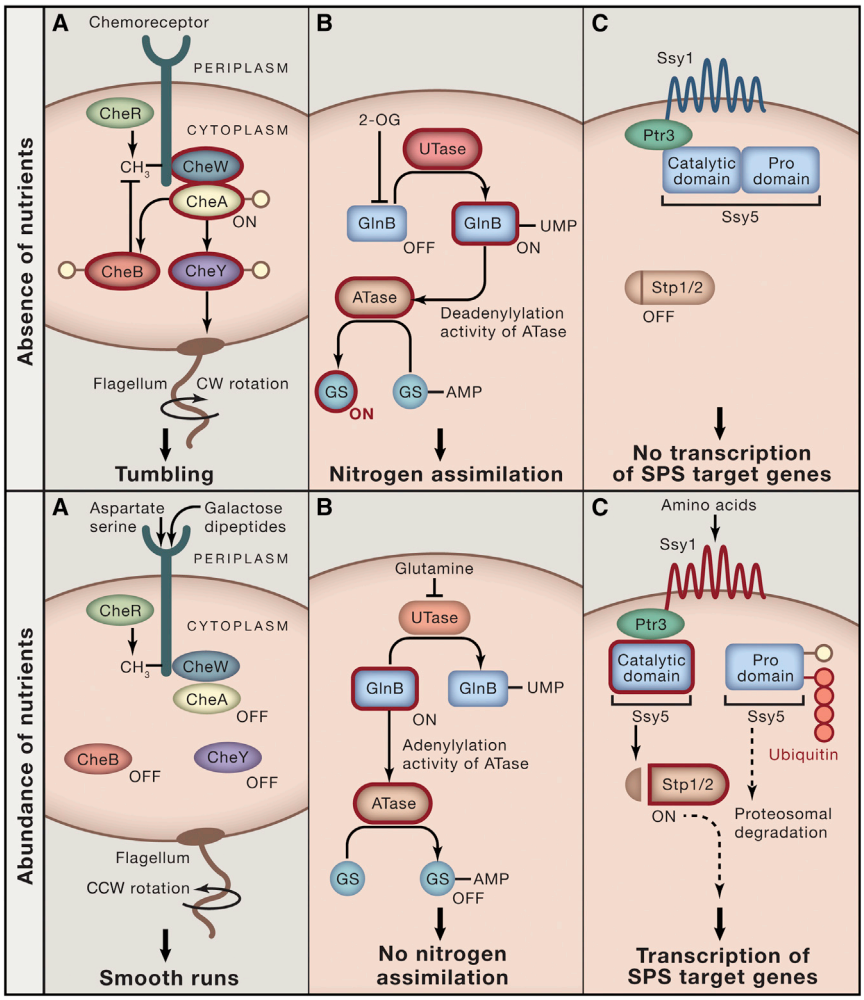

Figure 2. Select Sensing Pathways in Unicellular Organisms

(A) Chemotaxis in *E. coli*. In the absence of nutrients, the chemoreceptor activates the CheA kinase associated with CheW. CheA in turn phosphorylates and activates two critical effectors: CheY, which promotes clockwise rotation in the flagellar motor and random tumbling motions, and CheB, a demethylase involved in the adaptation process, which counteracts CheR, the constitutive methylase. Conversely, the presence of nutrients suppresses this pathway, and the default counterclockwise rotation of the rotor ensues to yield smooth runs.

(B) PII proteins in alpha proteobacteria. This tightly regulated protein family serves to control the adenylylation state and activity of glutamine synthetase (GS). When nitrogen is absent, the precursor of nitrogen assimilation reactions, 2-OG, accumulates, binds to, and inhibits the unmodified PII protein GlnB, which is unable to stimulate the adenylylation reaction of ATase. The unmodified and active form of GS accumulates. When nitrogen is abundant, glutamine levels are high, and this molecule binds and inhibits UTase, which permits the unmodified form of GlnB to accumulate and promote GS inhibition, by activating the adenylylation of GS by ATase.

(C) SPS pathway in *S. cerevisiae*. Extracellular amino acids bind directly to Ssy1, a transceptor with homology to amino acid permeases but lacking transport activity, to activate the SPS (Ssy1-Ptr3-Ssy5) pathway. Amino acids bind to Ssy1 to stimulate a conformational change in Ssy5, resulting in the phosphorylation and subsequent ubiquitin-mediated degradation of its inhibitory pro-domain. Ptr3 acts as an adaptor to mediate this process. Release of the catalytic domain of Ssy5 permits it to cleave the latent transcription factors Stp1 and Stp2, which translocate to the nucleus to activate transcription of genes involved in amino acid transport and metabolism.

Figure 2C adapted from Conrad et al., 2014.

to and activate the adenylyltransferase (ATase) enzyme (encoded by *glnE)* to adenylylate GS (Brown et al., 1971; Stadtman, 2001). The uridylylated form, on the other hand, stimulates the deadenylylation activity of ATase (Jaggi et al., 1997).

How nitrogen is sensed is complex and involves two sensors, the PII proteins themselves as well as the uridylyltransferase (UTase) enzyme (encoded by *glnD*) that uridylylates the PII proteins. Under conditions of nitrogen excess, UTase directly binds to and is inhibited by glutamine, which is at high levels when nitrogen is abundant (Jiang et al., 1998a). The glutamine-bound UTase is unable to uridylylate GlnB, which accumulates in its unmodified form and therefore allosterically activates the adenylylation activity of ATase (Adler et al., 1975; Mangum et al., 1973). This leads to the accumulation of adenylylated, and therefore inactive, GS. During nitrogen deprivation, however, 2-OG levels increase and the binding of 2-OG to unmodified GlnB allosterically inhibits its ability to activate adenylylation by ATase (Jiang et al., 1998c). The GlnB homotrimer has three 2-OG-binding sites, the first of which binds 2-OG with micromolar affinity, well below its physiological concentration of 0.1–0.9 mM. Because the binding of 2-OG to GlnB exhibits high negative cooperativity, the second and third sites are occupied only at the high 2-OG concentrations that occur upon nitrogen deprivation. Only upon binding multiple molecules of 2-OG does unmodified GlnB adopt a conformation in which it is unable to activate the adenylylation activity of ATase, leaving GS unmodified and thus active (Jiang et al., 1998a, 1998b, 1998c) (Adler et al., 1975; Mangum et al., 1973). While high levels of 2-OG inhibit the activity of unmodified GlnB, uridylylated GlnB is unaffected by high levels of this metabolite. GlnK, a PII protein that is highly similar to GlnB, also regulates AmtB, an ammonia transporter, in response to nitrogen availability, by binding to and inhibiting the transporter under high glutamine levels (Coutts, 2002; van Heeswijk et al., 1996).

While we have focused on the implications of 2-OG binding to the PII proteins, they are also well appreciated to bind ATP, which acts synergistically with 2-OG at low concentrations and is necessary for both GlnB and uridylylated GlnB to be able to stimulate the adenylylation and deadenylylation reactions, respectively, of ATase (Jiang et al., 1998c). While GlnB binds ATP with micromolar affinity and free ATP concentrations in the cell can be as high as 1 mM (Jiang and Ninfa, 2007; Jiang et al., 1998a), ATP binding, like that of 2-OG, displays negative cooperativity (Jiang and Ninfa, 2007). The first crystal structure

of GlnK in complex with AmtB showed that ADP could bind in place of ATP (Conroy et al., 2007), leading to the hypothesis that PII proteins may also act as energy sensors by responding to the ratio of ADP to ATP. However, more recent data suggest that additional work is needed to validate this hypothesis (Bennett et al., 2009; Chapman et al., 1971; Huergo et al., 2013; Radchenko et al., 2013; Zhang et al., 2009). In addition to roles in sensing nitrogen and perhaps energy, some PII proteins may also be carbon sensors (Feria Bourrellier et al., 2010; Huergo et al., 2013).

For several reasons, the sensing system anchored by the PII proteins is fascinating. First, two separate sensors detect two distinct metabolites—one that represents nitrogen depletion (2-OG) and the other abundance (glutamine). Second, because 2-OG binding exhibits negative cooperativity, the PII proteins are sensitive to differential flux through the nitrogen assimilation pathway rather than acting as binary switches. Lastly, the PII protein pathway is a good example of a particular sensing strategy: instead of directly sensing, like chemoreceptors, the nutrient of interest, it senses metabolites involved in nitrogen assimilation, thus providing specificity to this process over other ammonium-using reactions.

Eukaryotes

Nutrient-Sensing Systems Unique to Yeast

In this section, we discuss several pathways that sense extracellular nutrients and are specific to yeast, including those controlled by Ssy1, MEP2, Snf3, and Rgt2. Like bacterial chemoreceptors, these transmembrane proteins sense a diverse set of nutrients, including amino acids, ammonium, and glucose. They connect the status of the external world to varied intracellular processes—from the expression of transporters to the formation of pseudohyphae. Unlike bacterial chemoreceptors, Ssy1, MEP2, Snf3, and Rgt2 are homologous to nutrient transporters and are an important class of sensors sometimes termed transceptors. Many of these transceptors play key roles in allowing yeast to decide which nutrient to uptake and utilize when many are available and thus ensure an optimal growth rate.

MEP2: A Putative Extracellular Ammonium Sensor

Yeast evaluate and respond to a diverse set of nitrogen-containing compounds and have a hierarchical preference for nitrogen sources. In a process termed nitrogen catabolite repression, the presence of desired sources such as ammonium and glutamine represses the transcription of genes involved in scavenging and metabolizing poor sources such as proline (Zaman et al., 2008). Yeast can utilize ammonium as their sole source of nitrogen and assimilate it via biochemical reactions akin to those described in prokaryotes: glutamate dehydrogenase transaminates α-ketoglutarate to produce glutamate, which glutamine synthase uses with ammonium to make glutamine (Marini et al., 1994).

S. cerevisiae trigger a dimorphic transition under limiting ammonium conditions. Diploid cells form pseudohyphae that extend from the colony into the surrounding medium (Gimeno et al., 1992), permitting normally sessile yeast colonies to forage for nutrients at a distance from their colonization point (Gimeno et al., 1992). For this metamorphosis to take place, the lack of ammonium outside the cell must be sensed and transduced to downstream signaling pathways that control filamentous growth. MEP2 is proposed to mediate the sensing role in this pathway (Lorenz and Heitman, 1998).

The MEP (methylamine permease) proteins are members of a family of ammonium channels conserved from bacteria to animals (Marini et al., 1997a; Siewe et al., 1996), though their role in physiology has diverged in metazoans, as discussed below. In yeast, there are three MEP proteins, the most divergent being MEP2 (Marini et al., 1997b, 1994), and all three can uptake extracellular ammonium ions into cells. Of the three, MEP2 has the highest affinity, with a K_m of 1–2 uM, which contrasts with that of MEP3, which is 1–2 mM (Marini et al., 1997b). Given the dual role of MEP2 in detecting low ammonium concentrations in the environment and scavenging them for use as a nitrogen source, it makes sense that the highest-affinity transporter of the three evolved to be the sensor. Under low or absent ammonium, MEP2 is essential for pseudohyphal growth and is expressed on the plasma membrane (Dubois and Grenson, 1979; Rutherford et al., 2008) (Figure 2C). When ammonium is plentiful, MEP2 is internalized and targeted for degradation (Marini et al., 1997b; Zurita-Martinez et al., 2007).

Substantial evidence supports the notion that MEP2 is an ammonium sensor that controls pseudohyphal growth upon ammonium deprivation (Lorenz and Heitman, 1998). First, MEP2, but not MEP1 or MEP3, is required for pseudohyphal formation under these conditions, and its first intracellular loop is critical for this action (Lorenz and Heitman, 1998). Later mutagenesis studies revealed that a transport-deficient MEP2 prevents pseudohyphal growth despite proper localization and expression (Marini et al., 2006). However, transport is necessary, but not sufficient, for ammonium sensing, as there are transport-proficient but signaling-defective MEP2 mutants (Rutherford et al., 2008). The identity of the proteins that MEP2 engages to induce signaling remains unknown. Current evidence points to the involvement of GPA2, a G protein alpha subunit, and RAS2 in increasing cAMP levels to activate protein kinase A (PKA) in response to the absence of ammonium (Gimeno et al., 1992; Kübler et al., 1997; Lorenz and Heitman, 1997, 1998; Van Nuland et al., 2006).

As a transceptor, passage of ammonium through MEP2 is likely to induce a conformational change that enables it to interact with downstream effectors that signal pseudohyphal growth. Conformational changes have been observed in the bacterial homolog of MEP2, AmtB, but these remain to be linked to ammonium sensing (Andrade et al., 2005; Khademi et al., 2004; Zheng et al., 2004).

While ammonium is a valuable nitrogen source for bacteria, fungi, and plants, at high levels it is cytotoxic to animals (Biver et al., 2008). Therefore, in animals, ammonium transport is essential for its excretion, and MEP-like proteins have persisted throughout evolution (Marini et al., 1997a). Reflecting their functional conservation, human orthologs of MEP2, the erythroid specific Rh(rhesus)-type proteins, can transport ammonium in yeast (Marini et al., 1997a, 2000). Proteins of the Rh family are expressed in various organs and play critical roles in physiology. For instance, renal cortex cells excrete ammonium into urine via an Rh transporter (Biver et al., 2008; Garvin et al., 1988; Knepper et al., 1989). A role for these proteins as ammonium sensors has not been ascertained.

Extracellular Amino Acid Sensing: The SPS Pathway

Yeast coordinate signals from several major pathways to detect amino acids and alter gene expression and developmental decisions. While the GCN2 and TOR pathways discussed later respond to intracellular amino acids, the Ssy1-Ptr3-Ssy5 (SPS) pathway senses extracellular amino acids (Klasson et al., 1999). The SPS pathway is conserved in other yeast, such as *Candida albicans*, but not in higher eukaryotes (Davis et al., 2011), which have evolved distinct pathways for sensing extracellular amino acids (Conigrave et al., 2000; Cummings and Overduin, 2007) (Figure 1).

Ssy1 is a transporter-like protein in the plasma membrane of *S. cerevisiae* that functions, like MEP2, as a transceptor. Although it has sequence homology to amino acid permeases (AAP), it lacks transport activity and, unlike other AAPs, possesses a long N-terminal extension that is important for transmitting the availability of nutrients to downstream signaling elements (Bernard and André, 2001; Iraqui et al., 1999). Ssy1 forms a complex with Ptr3 and Ssy5 that, when amino acids are present, activates a signaling pathway that enhances the transcription of amino acid transport and metabolism genes (Conrad et al., 2014; Didion et al., 1998).

Ssy5 is an endoprotease composed of an inhibitory pro-domain and a catalytic domain (Abdel-Sater et al., 2004; Andreasson et al., 2006). Amino acid binding to Ssy1 on the extracellular side of the plasma membrane induces a conformational change in Ssy5 that leads to the phosphorylation and ubiquitin-mediated degradation of its pro-domain (Abdel-Sater et al., 2011; Omnus et al., 2011; Pfirrmann et al., 2010). This relieves the inhibition of the catalytic domain of Ssy5, which can cleave and activate Stp1 and Stp2, transcription factors that translocate into the nucleus to activate relevant genes. Ptr3, the third subunit of SPS, is essential for Ssy5 activation and is an adaptor that helps to transduce conformational changes from Ssy1 to Ssy5 upon amino acid binding and to bring the prodomain of Ssy5 into proximity with its kinase to facilitate its phosphorylation (Omnus and Ljungdahl, 2013). Evidence that SPS acts as a direct sensor of amino acids came from mutagenesis experiments demonstrating that certain Ssy1 mutants can alter the sensitivity of the SPS complex to extracellular amino acids (Poulsen et al., 2008). Interestingly, *S. cerevisiae* that harbor mutations in either Ptr3 or Ssy1 have increased vacuolar pools of basic amino acids (Klasson et al., 1999). This observation suggests that, in the absence of a signal relaying the presence of extracellular amino acids, yeast attempt to increase their vacuolar stores of amino acids, perhaps allowing them to be more independent of extracellular amino acid availability. Ssy1 is an interesting variant of the transceptor class of sensors because, unlike MEP2, it does not retain transport activity.

Snf3 and Rgt2: Extracellular Glucose Sensors

In addition to sensing extracellular amino acids, *S. cerevisiae* also detect extracellular glucose. Fermentation of this hexose yields the energy and carbon precursors needed to fuel growth, and glucose rapidly stimulates restructuring of the transcriptome to permit yeast to take full advantage of its presence (Zaman et al., 2008). In a process termed carbon catabolite repression, glucose and fructose repress processes involved in the metabolism of less-preferred carbon sources, with this repression occurring primarily at the transcriptional level (Gancedo, 1998; Santangelo, 2006). Here, we discuss the glucose-sensing pathway that regulates Rgt1, a transcription factor, and is necessary for glucose utilization. In the absence of glucose, the Snf1 (AMPK) pathway discussed later is essential for the use of less-preferred carbon sources (Zaman et al., 2008).

Under glucose limitation, Rgt1, in complex with the Ssn6-Tup1 repressor and the Mth1 and Std1 co-repressors, binds to the promoters of hexose transporter genes (*HXT*) and inhibits their transcription (Kim et al., 2003; Lakshmanan et al., 2003; Ozcan and Johnston, 1995; Polish, 2005; Theodoris et al., 1994; Tomas-Cobos and Sanz, 2002). Glucose binds to two transporter-like glucose sensors, Snf3 and Rgt2, which are needed to activate *HXT* expression. Snf3 senses low glucose concentrations and elevates the transcription of high-affinity hexose transporter genes while Rgt2 senses high glucose levels and promotes low-affinity hexose transporter expression (Bisson et al., 1987; Ozcan et al., 1996). Glucose binding to Snf3 and Rgt2 recruits Mth1 and Std1, through an unknown mechanism, to the plasma membrane, where they are subsequently phosphorylated, ubiquitylated, and degraded (Conrad et al., 2014; Flick, 2003; Kim et al., 2006; Moriya and Johnston, 2004; Schmidt et al., 1999). Without its co-repressors, Ssn6-Tup1 also dissociates from Rgt1 (Roy et al., 2013), leaving it free to be phosphorylated by the cAMP-dependent protein kinase (PKA) and to become a transcriptional activator of the *HXT* genes (Jouandot et al., 2011).

The Snf3/Rgt2 pathway represents yet another example of a nutrient-sensing pathway controlled by transceptors. In addition, analogous to the regulation of the PII proteins by 2-OG, the Snf3/Rgt2 pathway senses varied glucose levels rather than behaving like an on-off switch. Unlike the PII proteins, which use negative cooperativity between 2-OG-binding sites to allow for graded responses, the Snf3/Rgt2 pathway utilizes two separate sensors, with different affinities for the nutrient of interest.

Nutrient-Sensing Pathways Conserved from Yeast to Mammals

In this section, we highlight the AMPK, GCN2, and TOR pathways, which are conserved, at least in part, from yeast to man. In multicellular organisms, evolution has adapted the architectures of these pathways so they can sense hormonal cues in addition to the nutrients that the pathways detect in yeast. Given their roles in sensing essential nutrients, like amino acids and glucose, it is perhaps not surprising that the three pathways regulate feeding behavior.

Lastly, we briefly discuss how the emergence of the vacuole/lysosome in eukaryotes and its use as a storage depot for nutrients in fungi (Klionsky et al., 1990; Li and Kane, 2009), and likely mammals, has led to a need to sense its contents, which is a recently appreciated obligation of the TOR pathway.

AMPK: A Eukaryotic Fuel Gauge

A key event in the emergence of eukaryotes and their diversification and increase in complexity was the engulfment of oxidative bacteria, the predecessors of mitochondria. It has been argued that prokaryotes lack the energetic resources to maintain large amounts of regulatory DNA, but that the acquisition of mitochondria nearly 4 billion years ago alleviated the pressure to remove

excess DNA and permitted eukaryotes to explore hundreds of thousands-fold more genes (Lane and Martin, 2010).

Eukaryotes must sense their cellular energy balance and relay it to mitochondria and other parts of the cell that help maintain energy homeostasis (Hardie et al., 2012). A key energy sensor is the serine/threonine-directed AMP-activated protein kinase (AMPK), which regulates many catabolic and anabolic processes in response to energy levels (Gowans and Hardie, 2014).

AMPK was initially discovered in mammals as a kinase that phosphorylates and inactivates acetyl-CoA carboxylase (ACC) and HMG-CoA reductase, rate-limiting enzymes in fatty acid, and cholesterol synthesis, respectively (Carling et al., 1989; Carling and Hardie, 1986). The *S. cerevisiae* homolog of AMPK, SnfI (sucrose nonfermenting), had been found earlier in a screen for yeast that failed to grow on nonfermentable carbon sources, but it was not recognized as a homolog of AMPK until later (Carlson et al., 1981; Mitchelhill et al., 1994; Woods et al., 1994). AMPK orthologs have also been identified in plants and are referred to as SNF-1-related kinases (SnRK1). SnRK1 from rye endosperm can complement yeast *snf1* mutants, highlighting the conserved function of AMPK (Alderson et al., 1991).

In response to decreasing energy levels, AMPK and Snf1 phosphorylate substrates that activate processes that generate ATP and inhibit those that consume it. The conservation of the pathway throughout evolution is apparent in the high degree of similarity between the key effectors of AMPK and Snf1. For instance, both AMPK and Snf1 control glucose-linked processes. Snf1 inactivates the Mig1 transcriptional repressor, relieving glucose-repressed genes (Papamichos-Chronakis et al., 2004). Analogously, AMPK stimulates glucose uptake and glycolysis and inhibits glycogen synthesis (Yuan et al., 2013). Additional key effectors include the TORC1 and mTORC1 complexes, which function as master regulators of growth in yeast and mammals, respectively, and are discussed below. By regulating mTORC1 and TORC1, AMPK and Snf1 govern the switch between anabolism and catabolism to maintain metabolic homeostasis. In mammals, AMPK inhibits mTORC1 via two mechanisms. First, it phosphorylates and activates the TSC2 GTPase-activating protein, a major inhibitor of the pathway (Inoki et al., 2003b). Second, it phosphorylates raptor, a subunit of mTORC1, resulting in 14-3-3 binding and inhibition of mTORC1 kinase activity (Gwinn et al., 2008; Inoki et al., 2003b). Likewise, Snf1 has also been proposed to phosphorylate critical subunits of TORC1 (Braun et al., 2014). Aside from these well-characterized targets, AMPK and Snf1 likely have hundreds of additional substrates that control a wide range of processes (Hardie et al., 2012; Mihaylova and Shaw, 2011).

How do energy levels regulate AMPK? AMPK binds adenine nucleotides to sense the ratio of ADP or AMP to ATP, a critical barometer of the energy state of the cell. In times of nutrient abundance, this ratio is low. Upon energetic stress, the ratio rises as ATP levels drop with a concomitant rise in ADP, which is converted to AMP due to high cytosolic adenylate kinase activity (Hardie and Hawley, 2001). As opposed to ATP levels, AMP and ADP levels are more sensitive indicators of energy status; a 2-fold drop in ATP levels reflects a 50-fold rise in AMP (Hardie and MacKintosh, 1992). Furthermore, despite the millimolar concentrations of cellular ATP, a significant proportion of it is in complex with Mg^{2+} and does not bind well to AMPK (Xiao et al., 2011).

The mechanism of adenine nucleotide regulation of AMPK has been extensively characterized. AMPK is a trimeric complex composed of α kinase, β carbohydrate binding, and γ regulatory subunits (Kemp, 2004; Scott et al., 2004). There are theoretically four nucleotide-binding sites in the γ subunit, though one remains empty and another constitutively binds AMP (Xiao et al., 2007). A tripartite mechanism controls AMPK in mammalian cells. First, AMPK binds AMP and undergoes a conformational change that enhances the ability of the kinases LKB1 and CaMKKKB to phosphorylate and activate it. Second, AMP binding to AMPK protects it against dephosphorylation by currently unidentified phosphatases. Third, AMP further allosterically activates the kinase up to 13-fold (Carling et al., 1989; Gowans and Hardie, 2014). ATP binding antagonizes all of these effects (Corton et al., 1995). As a result of the two nucleotide-binding sites, both of which can bind AMP, ADP, or ATP, AMPK regulation is graded in response to energy status, just like PII protein function is in response to 2-OG. As AMP levels rise under extreme energetic stress, AMP binds both sites to maximally activate AMPK.

Recent studies have uncovered conservation between the regulatory mechanisms of fungal and plant AMPK homologs and those of the mammalian kinase. In yeast, SnfI is also heterotrimeric, binds nucleotides, and is regulated by opposing kinases and phosphatases (Hong et al., 2003, 2005; Jin et al., 2007; Sutherland et al., 2003; Townley and Shapiro, 2007). However, unlike AMPK, it appears that ADP, not AMP, promotes phosphorylation of Snf1 by inhibiting its dephosphorylation, and AMP does not allosterically activate Snf1 (Mayer et al., 2011; Mitchelhill et al., 1994; Woods et al., 1994). Hence, in yeast, there is a bipartite mechanism of activation, with ADP playing a prominent role.

With the onset of multicellularity, physiological processes evolved in metazoans that maintain homeostasis for the organism as a whole, and AMPK acquired new modes of regulation. Specifically, hormones and cytokines enable the nutrient-sensing organs of multicellular organisms to communicate the nutritional state to other organs to elicit tissue-specific responses. The coordinated actions of leptin, insulin, and ghrelin, among others, regulate the organismal response to nutrients, or lack thereof, and are well appreciated to regulate AMPK. Upon food consumption, blood glucose levels rise and pancreatic beta cells release insulin, which promotes anabolic and inhibits catabolic processes in many tissues. These effects are mediated, in part, through Akt, a kinase that inhibits AMPK by phosphorylating it at $Ser^{485/491}$, and antagonizing LKB1-mediated Thr^{172} phosphorylation, which normally activates AMPK (Horman et al., 2006).

Many nutrient-regulated hormones signal to the brain to control feeding behavior. Under fasting or starvation conditions, enteroendocrine cells of the stomach release ghrelin, which signals hunger. Conversely, during feeding, adipocytes release leptin, which signals satiety. These hormones alter the activity of neuronal circuits in the hypothalamic arcuate nucleus, the appetite control center of the brain (Dietrich and Horvath, 2011; Hardie et al., 2012; Pinto et al., 2004). Several studies point

to a role for AMPK in the control of feeding. Ghrelin activates AMPK in the hypothalamus and leads to a subsequent increase in food intake. Overexpression of a dominant-negative form of AMPK in the hypothalamus restrains feeding while direct injection of pharmacological AMPK activators results in hyperphagia (Andersson et al., 2004; Minokoshi et al., 2004). These effects are likely mediated through modulation of AMPK in presynaptic neurons that impinge on neurons critical for feeding control (Gowans and Hardie, 2014; Yang et al., 2011). Ghrelin likely binds GHSR1, a G-protein-coupled receptor in the presynaptic neuron, triggering the release of Ca^{2+} that stimulates CaMKKK to activate AMPK (Yang et al., 2011). Meanwhile, leptin may function in a manner similar to insulin by activating the PI3K-Akt axis and controlling the phosphorylation state of AMPK (Dagon et al., 2012). Therefore, as complex feeding behaviors arose in multicellular organisms, AMPK was coopted to function in neuronal circuits to control intake of food.

GCN2: A Sensor of Amino Acid Deprivation

Alongside the SPS pathway, which senses extracellular amino acids in yeast, eukaryotes evolved a parallel pathway to detect intracellular amino acid levels: the general amino acid control non-derepressible 2 (GCN2) pathway. GCN2 senses the uncharged tRNAs that accumulate upon amino acid deprivation. GCN2 attenuates translation, which not only consumes amino acids, but is also one of the most energy-demanding processes in the cell (Lane and Martin, 2010).

While GCN2 is found only in eukaryotes, the use of uncharged tRNAs to signal amino acid deprivation is conserved to bacteria. Upon amino acid starvation in *E. coli*, uncharged tRNAs bind directly to ribosomes, leading to the production of the odd nucleotides guanosine tetraphosphate and guanosine pentaphosphate (Cashel and Gallant, 1969). These nucleotides repress the synthesis of stable RNAs (rRNA and tRNA) and activate amino acid biosynthetic genes to promote survival in a process referred to as the stringent response (Srivatsan and Wang, 2008).

In yeast, GCN2 is dedicated to sensing uncharged tRNAs (Hinnebusch, 1984). Under conditions of amino acid limitation or a defect in an amino acyl tRNA synthetase, *S. cerevisiae* upregulate the transcription of genes involved in amino acid biosynthesis, a process termed general amino acid control (Hinnebusch, 1988; Hinnebusch, 2005; Wek et al., 1995). When present, uncharged tRNAs bind to the histidyl tRNA synthetase-like domain of GCN2, which lacks residues critical for synthetase activity and histidine-specific binding, thus enabling GCN2 to respond to a variety of uncharged tRNAs (Wek et al., 1989; Wek et al., 1995). The binding triggers GCN2 homodimerization (Narasimhan et al., 2004) and autophosphorylation (Diallinas and Thireos, 1994), allowing it to phosphorylate and inhibit its only known substrate, eukaryotic initiation factor 2a (eIF2a) (Dever et al., 1992). This phosphorylation prevents efficient translation initiation of most mRNAs by limiting the pool of ternary complex, which consists of eIF2, GTP, and methionyl initiator tRNA and is required for translation initiation (Abastado et al., 1991; Dever et al., 1992; Hinnebusch, 1993).

While most mRNAs are translationally repressed upon amino acid deprivation, the mRNA encoding the GCN4 transcription factor is derepressed so that GCN4 can accumulate and activate the expression of genes that promote amino acid biosynthesis (Abastado et al., 1991; Dever et al., 1992; Hinnebusch, 1993). A cluster of four upstream open reading frames (uORFs) in the 5′ untranslated region of the GCN2 mRNA permits this unique regulation (Hinnebusch, 2005). Under nutrient-replete conditions, a ternary complex forms at the first uORF. It then dissociates and another forms at the subsequent uORFs, thus preventing translation of the main ORF. However, upon starvation, ternary complex formation is delayed, and rebinding at latter uORFs is reduced. Larger proportions of preinitiation complexes bypass the uORFs and form ternary complexes before reaching and translating the main ORF (Abastado et al., 1991; Hinnebusch, 1984; Mueller and Hinnebusch, 1986).

In mammals, GCN2 pathway architecture is reminiscent of that in yeast (Berlanga et al., 1999; Sood et al., 2000), as it is activated by a limitation in an essential amino acid or inhibition in the synthesis of a nonessential amino acid. uORFs also regulate the translation of the mammalian GCN4 ortholog, ATF4, a basic leucine zipper transcription factor (Vattem and Wek, 2004). ATF4 induces a cascade of transcriptional regulators that contribute to a gene expression program that modulates apoptosis, autophagy, and amino acid metabolism, including upregulation of select amino acyl tRNA synthetases and amino acid transporters (B'chir et al., 2013; Bunpo et al., 2009; Harding et al., 2000, 2003; Krokowski et al., 2013). Deletion of GCN2 in mice decreases their viability during prenatal and postnatal development under conditions of nutritional stress, most notably leucine deprivation (Zhang et al., 2002). When challenged with a leucine-free diet for several days, GCN2 null mice lose more body weight than wild-type counterparts, and a significant proportion perish (Anthony et al., 2004).

Like AMPK, GCN2 has acquired a critical role in controlling feeding behavior in animals. When rodents encounter a food source that lacks a single essential amino acid, they recognize this deficiency and reduce the intake of the imbalanced food (Koehnle et al., 2003). GCN2 activity in the anterior piriform cortex (APC) mediates this behavior. Injection of amino acid alcohol derivatives such as threoninol into the APC increases the levels of uncharged tRNAs and promotes the rejection of diets low in the corresponding amino acid (Hao et al., 2005). Furthermore, mice with full body or brain-specific GCN2 deletions fail to reject food depleted of leucine or threonine, unlike wild-type counterparts (Hao et al., 2005; Maurin et al., 2005). At the signaling level, ingestion of a meal imbalanced in amino acid composition rapidly elevates phosphorylated eIF2a in APC neurons of wild-type, but not GCN2 null, mice (Hao et al., 2005; Maurin et al., 2005; Gietzen et al., 2004). The need to adapt feeding behavior to changes in nutrient levels is by no means restricted to animals. *Drosophila* also sense changes in dietary amino acids and reduce their intake of foods deficient in essential amino acids (Bjordal et al., 2014; Ribeiro and Dickson, 2010; Toshima and Tanimura, 2012; Vargas et al., 2010). As in animals, GCN2 plays a critical role within neuronal circuits to mediate this rejection by repressing GABA signaling within dopaminergic neurons of the brain (Bjordal et al., 2014). Together, these findings point to a role for the detection of uncharged tRNAs by GCN2 in controlling circuits in flies and animals that protect against the consumption of imbalanced food sources.

A separate pathway discussed below, TORC1/mTORC1, evolved in parallel to the GCN2 pathway to sense the availability of intracellular amino acids. The mechanisms for crosstalk between the TORC1/mTORC1 and GCN2 pathways were acquired at least twice during evolution, albeit in opposing directions. While in yeast GCN2 lies downstream of TORC1, it functions upstream of mTORC1 in mammals (Anthony et al., 2004; Cherkasova and Hinnebusch, 2003; Kubota et al., 2003; Staschke et al., 2010).

TOR/mTOR: Master Regulators of Growth

Nutrient availability strongly influences the growth of all organisms, and starvation conditions can alter developmental programs in both unicellular and multicellular organisms (Oldham et al., 2000). When faced with nutritional limitation, *S. cerevisiae* exit the mitotic cycle and enter a stationary phase (Zaman et al., 2008); *C. elegans* persist for several months in a state of stasis known as dauer larvae (Klass and Hirsh, 1976); and *Drosophila* postpone their development (Edgar, 2006). Despite the diversity of these organisms, one common pathway, anchored by the target of rapamycin (TOR) kinase, regulates entry into these alternative states in response to environmental cues. Unlike the GCN2 and AMPK pathways, the TOR pathway is unique in that it integrates not a few but many diverse inputs, particularly in mammals. In fact, GCN2 and AMPK both feed into TOR to convey amino acid or energy deprivation, respectively. While we focus on the two major inputs that control mTOR activity—nutrients and growth factors—numerous additional cues converge on it (Laplante and Sabatini, 2012).

The study of TOR began several decades ago with the isolation of a potent antifungal compound from the soils of Rapa Nui, more commonly known as Easter Island. This macrolide, named rapamycin in deference to its site of discovery, garnered clinical and research interest because of its powerful immunosuppressive and anti-proliferative qualities (Morris, 1992; Segall et al., 1986). Genetic studies in yeast led to the identification of TOR1 and TOR2 as key genes mediating rapamycin sensitivity (Cafferkey et al., 1993; Kunz et al., 1993), and biochemical work in mammals revealed the mTOR (mechanistic target of rapamycin) protein as the direct target of rapamycin (Brown et al., 1994; Sabatini et al., 1994; Sabers et al., 1995). mTOR is a serine/threonine protein kinase that responds to a variety of environmental cues, including nutrient, energy, and growth factor levels, as well as diverse forms of stress, to regulate many anabolic and catabolic processes (Howell et al., 2013; Kim et al., 2013; Laplante and Sabatini, 2012).

Unlike most eukaryotes, *S. cerevisiae* encode two different TOR proteins, Tor1 and Tor2, which nucleate distinct multiprotein complexes (Helliwell et al., 1994; Loewith et al., 2002). TOR complex 1 (TORC1) consists of Tor1 bound to Kog1, Lst8, and Tco89 and promotes ribosome biogenesis and nutrient uptake under favorable growth conditions. Inhibition of TORC1 by nutrient starvation or rapamycin treatment leads to the activation of macroautophagy and nutrient- and stress-responsive transcription factors like GLN3, which is required for the use of secondary nitrogen sources (Jacinto et al., 2004; Wullschleger et al., 2006). TORC2 contains Tor2 bound to Avo1-3, Bit61, and Lst8; is largely rapamycin insensitive; and is thought to regulate spatial aspects of growth, such as cytoskeletal organization (Loewith et al., 2002; Reinke et al., 2004; Wedaman et al., 2003).

Mammals also have two mTOR-containing complexes but only one gene encoding mTOR. mTOR complex 1 (mTORC1) consists of raptor, mLST8, PRAS40, and Deptor (Laplante and Sabatini, 2012) and modulates mass accumulation through the control of many anabolic and catabolic processes, including protein, lipid, and nucleotide synthesis; ribosome and lysosome biogenesis; and autophagy. mTORC2 controls cell proliferation and survival and is further reviewed elsewhere (Jacinto et al., 2004; Oh and Jacinto, 2011; Sarbassov et al., 2004).

The connection between TORC1 and the response to the nutritional state emerged from observations in *S. cerevisiae*, *D. melanogaster*, and mammalian cells, where TOR inhibition leads to phenotypes akin to those observed under starvation (Barbet et al., 1996; Oldham et al., 2000; Peng et al., 2002; Zhang et al., 2000). Environmental amino acid levels were found to activate the mTORC1 pathway as measured by the phosphorylation of S6K1 and 4EBP1, two well-known mTORC1 substrates (Hara et al., 1998; Wang et al., 1998), and to signal independently of the growth factor input to mTORC1 (Hara et al., 1998; Nobukuni et al., 2005; Roccio et al., 2006; Smith et al., 2005; Svanberg and Moller-Loswick, 1996; Wang et al., 1998).

More recent work showing that the Rag GTPases are necessary and sufficient for mTORC1 to sense amino acids (Kim et al., 2008; Sancak et al., 2008) is beginning to reveal the logic of how the pathway integrates inputs from nutrients and growth factors. What has emerged is a bipartite mechanism of mTORC1 activation involving two distinct small GTPases: first, the control of mTORC1 subcellular localization by nutrients through the Rag GTPases, and, second, the control of mTORC1 kinase activity by growth factors and energy levels through the Rheb GTPases (Zoncu et al., 2011). Both inputs are needed for full activation of mTORC1, as in the absence of either, the pathway is off.

The Rag GTPases exist as heterodimers of the related and functionally redundant RagA or RagB bound to RagC or RagD, which are also very similar (Hirose et al., 1998; Schürmann et al., 1995; Sekiguchi et al., 2001). Under nutrient-replete conditions, the Rag GTPases bind mTORC1 and promote its recruitment to the lysosomal surface, where its activator Rheb also resides (Buerger et al., 2006; Saito et al., 2005; Sancak et al., 2008) (Figure 3). The function of each Rag within the heterodimer is poorly understood, and their regulation is undoubtedly complex, as many distinct factors play key roles. A lysosome-associated molecular machine consisting of the Ragulator and vacuolar ATPase (v-ATPase) complexes regulates the Rag GTPases and is necessary for the sensing of amino acids by mTORC1 (Sancak et al., 2010; Zoncu et al., 2011). Ragulator binds the Rag GTPases to the lysosome and also has nucleotide exchange activity for RagA/B (Bar-Peled et al., 2012; Sancak et al., 2010), but the function of the v-ATPase in the pathway is unknown. Two GTPase-activating protein (GAP) complexes, which are both tumor suppressors, promote GTP hydrolysis by the Rag GTPases, with GATOR1 acting on RagA/B (Bar-Peled et al., 2013) and Folliculin-FNIP2 on RagC/D (Tsun et al., 2013). Lastly, a distinct complex called GATOR2 negatively regulates GATOR1 through an unknown mechanism

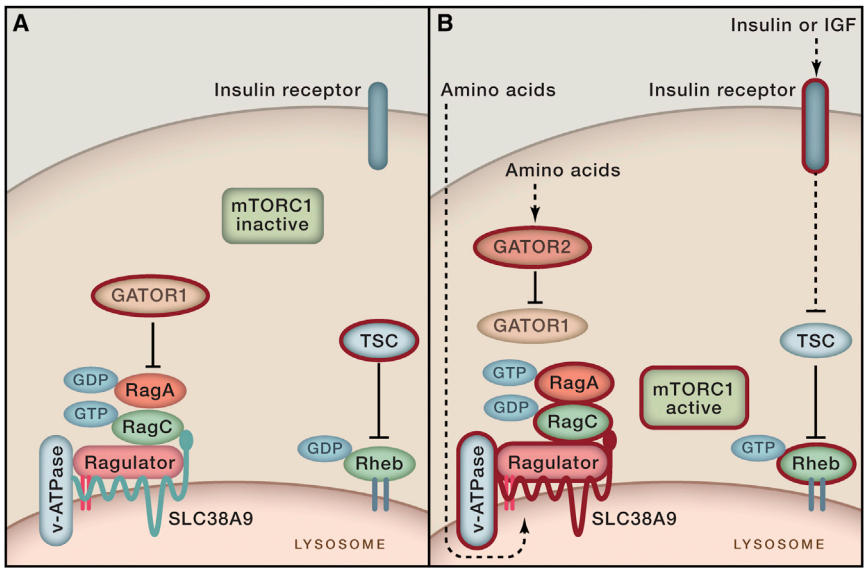

Figure 3. Nutrient Sensing by the TOR Pathway

(A) In the absence of amino acids and growth factors, mTORC1 is inactive. This is controlled by two separate signaling pathways. First, in the absence of amino acids, GATOR1 is an active GAP toward RagA, causing it to become GDP bound. In this state, mTORC1 does not localize to the lysosomal surface. Second, in the absence of insulin or growth factors, TSC is an active GAP toward Rheb and stimulates it to be GDP bound.

(B) In the presence of amino acids and growth factors, mTORC1 is active. Amino acids within the lysosome signal through SLC38A9 to activate the amino acid sensing branch. Ragulator is active, causing RagA to be GTP bound. This binding state is reinforced by the fact that GATOR1 is inactive in the presence of amino acids, as GATOR2 inhibits it. The Rag heterodimer in this nucleotide conformation state recruits mTORC1 to the lysosomal surface. In addition, the presence of growth factors activates a pathway that inhibits TSC, leaving Rheb GTP bound. In this state, Rheb activates mTORC1 when it translocates to the lysosomal surface.

(Bar-Peled et al., 2013). The Sestrins were recently identified as GATOR2-interacting proteins that negatively regulate mTORC1 (Chantranupong et al., 2014; Parmigiani et al., 2014).

Growth factors and energy levels regulate the Rheb input to mTORC1 (Inoki et al., 2003a; Long et al., 2005) through a heterotrimeric complex comprised of the tuberous sclerosis complex (TSC) proteins TSC1, TSC2, and TBC1D7, which together act as a GAP for Rheb (Brugarolas et al., 2004; Dibble et al., 2012; Garami et al., 2003; Inoki et al., 2003a; Long et al., 2005; Sancak et al., 2008; Saucedo et al., 2003; Stocker et al., 2003; Tee et al., 2002). Not all unicellular organisms encode all components of the TSC axis. For instance, *S. cerevisiae* only have a gene for Rheb, and it is not required for growth or viability, unlike TOR itself, suggesting that it likely plays a diminished, if any, role in the TOR pathway in budding yeast (Urano et al., 2000). In contrast, *S. pombe*, which diverged from *S. cerevisiae* more than 400 million years ago, encode TSC1, TSC2, and Rheb (Rhb1), whose functions mirror their mammalian equivalents. Rhb1 is essential for growth, and it is negatively regulated by TSC1 and TSC2, whose loss results in defects in amino acid uptake and the nitrogen starvation response (Ma et al., 2013; Mach et al., 2000; Matsumoto et al., 2002; Nakashima et al., 2010; Urano et al., 2007; Uritani et al., 2006; van Slegtenhorst et al., 2004).

While in mammals, growth is intimately linked to amino acid availability, yeast are more concerned with the quality and abundance of nitrogen and can uptake and metabolize a host of nitrogen sources, including amino acids, which are deaminated to yield ammonia that will rapidly become ammonium in the cell. In yeast, the aforementioned SPS and GCN2 pathways directly or indirectly, respectively, sense amino acid levels, but the actual intracellular signal for TORC1 remains less clear (Broach, 2012). Early studies showed that TORC1 is a major regulator of the nitrogen catabolite repression program (Hardwick et al., 1999; Shamji et al., 2000), although later work emphasizes that TORC1 is likely not the sole player regulating this pathway (Broach, 2012). Further studies are needed to ascertain whether TOR is involved in the sensing of an as yet unidentified nitrogen source in yeast.

More recent evidence indicates that TORC1 is involved in amino acid signaling in yeast (Binda et al., 2009; De Virgilio and Loewith, 2006). TORC1 resides on the vacuole, the equivalent of the metazoan lysosome, although it does not shuttle on and off its surface in response to nutrient levels as it does in mammals (Binda et al., 2009). Homologs of the Rag GTPases, Gtr1 and Gtr2, exist in yeast and associate with a vacuolar docking complex consisting of Ego1 and Ego3, which has some structural similarity to Ragulator (Bun-Ya et al., 1992; Dubouloz et al., 2005; Gao and Kaiser, 2006; Kogan et al., 2010). Yeast also have GATOR1 and GATOR2 equivalents, called SEACIT and SEACAT (Panchaud et al., 2013a, b). SEACIT has been proposed to inhibit TORC1 in response to deprivation of sulfur-containing amino acids, such as methionine and cysteine, and controls glutamine synthesis and consumption (Laxman et al., 2013, 2014; Sutter et al., 2013). Although TORC1 has been posited to respond to amino acids, constitutively active Gtr1 does not make the TORC1 pathway completely resistant to leucine deprivation, unlike constitutively active RagA/B, which in mammals makes mTORC1 signaling resistant to total amino acid deprivation (Binda et al., 2009; Sancak et al., 2008; Efeyan et al., 2013). Furthermore, the Gtr GTPases are dispensable for growth on glutamine or ammonium (Stracka et al., 2014), and constitutively active Gtr1 fails to rescue the TORC1 signaling defect under ammonium deprivation (Binda et al., 2009). If amino acids signal to TORC1, the mechanisms of its activation are likely to be distinct from those through which amino acids activate mTORC1. For instance, orthologs of Sestrins do not exist in yeast, suggesting divergence in the regulation of the upstream components of the nutrient-sensing pathway.

While many components of the pathway upstream of mTORC1 have been identified, the identity of the amino acid sensor(s) remains elusive. Amino acid sensing could initiate

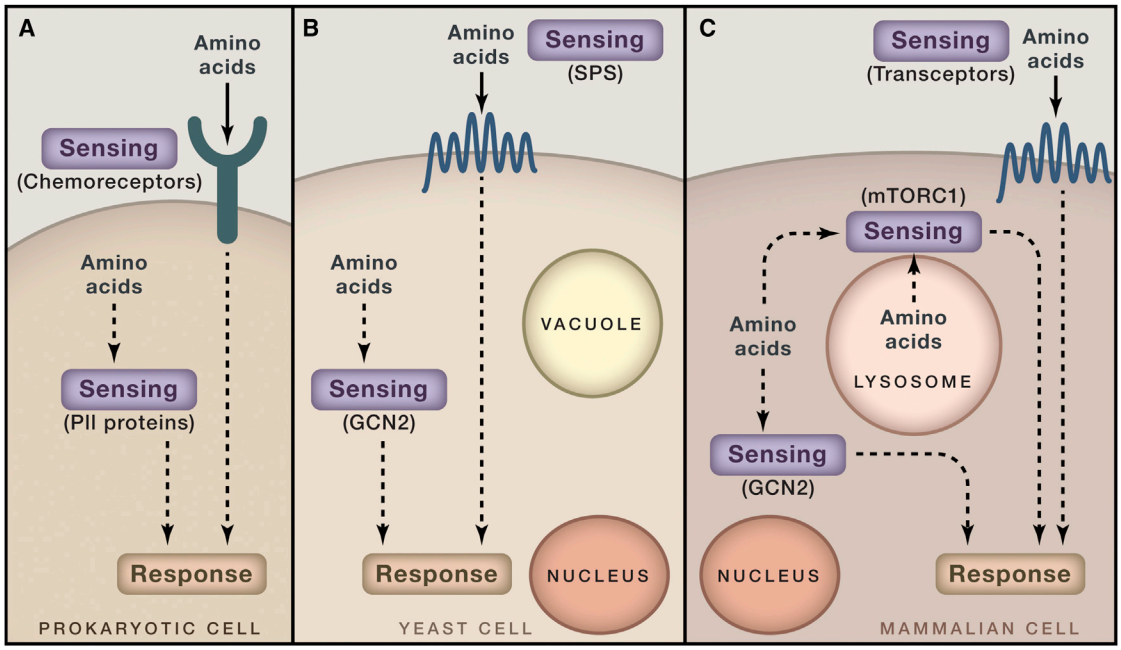

Figure 4. Evolution of Nutrient Sensing with the Emergence of Multicellularity and Compartmentalization

(A) Prokaryotes have two compartments that may contain amino acids: the extracellular space and the cytosol. Amino acid receptors such as the chemoreceptors Tsr and Tar can sense extracellular amino acids. A variety of sensors, including the PII proteins, can detect intracellular amino acids.

(B) Similar to prokaryotes, yeast sense extracellular amino acids via plasma membrane transporters, such as Ssy1. In addition, they sense cytosolic amino acid availability with sensors like GCN2. Unlike prokaryotes, however, eukaryotes have organelles such as the vacuole, an additional compartment that may contain amino acids. While it has not yet been established whether yeast directly sense amino acid levels within the vacuole, they do contain organelles that can act as alternate storage depots for nutrients and are therefore another potential compartment in which sensing may occur.

(C) In mammalian cells, there are three distinct compartments in which sensing may occur, similar to yeast. First, extracellular nutrients are sensed via transceptors, not discussed in detail in this Review. In addition, cytosolic amino acids are sensed by the GCN2 pathway. Finally, amino acids stored within the lysosome are sensed by the mTORC1 pathway.

from the extracellular, cytosolic, or lysosomal compartments (Figure 4). The presence of many mTORC1 pathway components on the lysosome suggests that this organelle is more than simply a scaffold surface for mTORC1 activation. Rather, there is the intriguing possibility that lysosomes act as storage sites for amino acids and that amino acid availability within this compartment is sensed by mTORC1. The storage of nutrients in vacuoles, which is established in yeast, may also occur in mammalian cells, as some studies suggest that certain amino acids, like arginine, are highly enriched in lysosomes relative to the cytosol (Harms et al., 1981). A cell-free assay revealed that the lysosome itself contains the minimal machinery needed for the amino-acid-mediated recruitment of mTORC1 to the lysosomal surface (Zoncu et al., 2011).

In an "inside-out" model of sensing, a lysosome-based transmembrane protein would be an alluring candidate amino acid sensor. One such protein is SLC38A9, a newly identified Ragulator-interacting amino acid transporter that resides in the lysosomal membrane and is required for arginine sensing by mTORC1 (Rebsamen et al., 2015; Wang et al., 2015). Like Ssy1 of the SPS pathway, SLC38A9 contains an N-terminal extension that appears necessary for the downstream signaling event (Bernard and André, 2001; Wang et al., 2015). In cells lacking SLC38A9, the mTORC1 pathway has a selective defect in sensing arginine, suggesting that SLC38A9 is an attractive candidate to be an arginine sensor (Wang et al., 2015). While the mechanism through which SLC38A9 regulates the mTORC1 pathway remains unknown, this transporter is the best candidate so far identified for reporting the contents of lysosomes to mTORC1 in the cytosol. It is very likely that, in addition to sensing lysosomal amino acids, mTORC1 will be found to also sense cytosolic amino acids and integrate information from both amino acid pools.

Perspectives

Nutrient sensors are of diverse structure and function, from membrane spanning transceptors like MEP2 and Ssy1 to the cytosolic kinases AMPK and GCN2. They can directly sense nutrients of interest, such as ammonium by MEP2, or indirectly via a metabolite, such as 2-OG by the PII proteins. While direct nutrient sensing will give a good reflection of its overall levels, indirect sensing strategies may allow the sensor to detect each nutrient under specific contexts, such as when flux through the GS/GOGAT pathway is low in the case of the PII proteins.

In eukaryotic cells, the vacuole/lysosome emerged as a nutrient storage compartment, and there is increasing evidence that a function of mTORC1 is to sense lysosomal contents and/or function. Although it is unclear whether this is true in yeast, the presence of TORC1 on the vacuolar surface suggests that it will also be the case in this organism, although it is unclear

whether a homolog of SLC38A9 exists. Many intriguing questions remain concerning nutrient sensing by mTORC1/TORC1. Does the pathway sense all amino acids, or are there particular single amino acids or combinations that are especially important? It is already known that leucine or arginine withdrawal inhibits mTORC1 signaling almost as well as total amino acid deprivation in a few cell lines in culture (Hara et al., 1998), but how true is this in vivo or in diverse cell types? Furthermore, could other Rag-independent mechanisms be involved in relaying amino acid signals to mTORC1? A recent work revealed that, in mammalian cells, glutamine, unlike leucine, signals to mTORC1 in a v-ATPase-dependent but Rag-GTPase-independent manner (Jewell et al., 2015). Are different amino acids differentially important in the cytosol versus lysosome? The lysosome is enriched for basic amino acids, hinting that these amino acids may matter more than others in sensing that initiates from this organelle (Harms et al., 1981), which is consistent with the specific defect of cells lacking SLC38A9 in sensing arginine (Wang et al., 2015). Finally, how well conserved the sensors are between organisms will hint at how different or similar the amino acid and nutrient inputs are that drive mTORC1/TORC1 signaling in diverse organisms.

ACKNOWLEDGMENTS

We thank Zhi-Yang Tsun for insightful input and Tom DiCesare for assistance with figures. This work was supported by grants from the NIH (R01 CA103866 and AI47389) and Department of Defense (W81XWH- 07-0448) to D.M.S. and by fellowship support from the NIH to L.C. (F31 CA180271) and to R.L.W. (T32 GM007753). D.M.S. is an Investigator of the Howard Hughes Medical Institute.

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Cell

Leading Edge
Review

Time for Food: The Intimate Interplay between Nutrition, Metabolism, and the Circadian Clock

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http://dx.doi.org/10.1016/j.cell.2015.03.015

The circadian clock, a highly specialized, hierarchical network of biological pacemakers, directs and maintains proper rhythms in endocrine and metabolic pathways required for organism homeostasis. The clock adapts to environmental changes, specifically daily light-dark cycles, as well as rhythmic food intake. Nutritional challenges reprogram the clock, while time-specific food intake has been shown to have profound consequences on physiology. Importantly, a critical role in the clock-nutrition interplay appears to be played by the microbiota. The circadian clock appears to operate as a critical interface between nutrition and homeostasis, calling for more attention on the beneficial effects of chrono-nutrition.

The approach to healthful eating proposed by Maimonides (1135–1204), a medieval Jewish philosopher and doctor also known as Rambam, has garnered followers from well beyond the grave. In his writings, the Rambam gave clear instructions regarding what, when, and how much people should eat in order to lead a healthy life. One of his well-known quotes is: ''Eat like a king in the morning, a prince at noon, and a peasant at dinner.''

Feeding behavior is a principal factor that plays a role in the organism's nutritional status. Eating schedules are predominantly dictated by an inherent timing mechanism, but in addition, are affected by food availability, hunger, and satiety and also by social habits and convenience. A large body of nutritional studies has extensively examined the effect of the quantity and quality of food ingested on the organism's well-being. Nowadays, it is widely accepted that these parameters are critical and that their alteration is associated with morbidity and mortality (e.g., high-fat diet). Evidence accumulated during recent years suggests that meal timing can affect a wide variety of physiological processes, including sleep/wake cycle, core body temperature, performance, and alertness. Moreover, it appears that feeding time has a dramatic effect on health and can be employed to prevent obesity and various other metabolic pathologies. Hence, ''chrono-nutrition'' refers to food administration in coordination with the body's daily rhythms. This concept reflects the basic idea that, in addition to the amount and content of food, the time of ingestion is also critical for the well-being of an organism.

Hitherto, as detailed in this Review, the vast majority of studies have focused on the effect of scheduled meals on metabolic pathologies such as obesity and diabetes. However, one can envision that the ''optimal'' feeding schedule might harbor wide medical benefits beyond metabolic syndrome. Future studies are expected to shed more light on the prospects of feeding timing in preventing morbidity and reducing mortality in relation to other pathologies such as aging.

Circadian Clocks and Metabolism

A wide array of physiological and metabolic variations depends on the time of the day, including sleep-wake cycles, feeding behavior, body temperature, and hormonal levels. The past two decades have witnessed a remarkable increase in our knowledge of how circadian (from the Latin words, *circa diem*, about a day) biology is controlled, both from physiological and molecular standpoints. We refer the interested reader to several detailed review articles on the subject (Asher and Schibler, 2011; Eckel-Mahan and Sassone-Corsi, 2013; Feng and Lazar, 2012). Briefly, circadian rhythms are controlled by molecular clocks, whose key features are (1) an input pathway that includes receivers for environmental cues and subsequently transmits them to the central oscillator; (2) a central oscillator that keeps circadian time and generates rhythm; and (3) output pathways through which the rhythms are conveyed and control various metabolic, physiological, and behavioral processes. Circadian clocks are uniquely characterized as entrainable, self-sustained, and temperature-compensated oscillators (Brown et al., 2012; Buhr and Takahashi, 2013; Dibner et al., 2010). The master or ''central'' clock is located in the hypothalamus, within a paired structure so-called the suprachiasmatic nucleus (SCN). The SCN contains 15–20,000 neurons, which have the remarkable feature of oscillating with a 24 hr based rhythm. Indeed, the SCN clock can function autonomously, without any external input, and can be reset in response to environmental cues (zeitgebers, or time givers) such as light. The SCN functions as an ''orchestra director'' for the ''peripheral clocks,'' thought to be present in all other tissues and cells in the body. Synchronization of peripheral clocks is essential to ensure temporally coordinated physiology and is achieved through yet-ill-defined pathways controlled by the master clock (Saini et al., 2011).

At the heart of the molecular network that constitutes the circadian clock are the core transcription factors CLOCK and

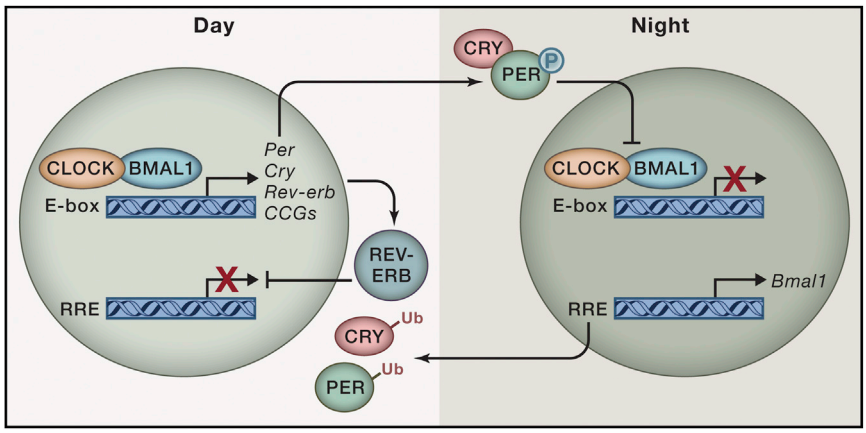

Figure 1. The Molecular Organization of the Circadian Clock

The transcriptional activators CLOCK and BMAL1 dimerize to stimulate the expression of many CCGs with E-box promoter elements in their promoters. CLOCK:BMAL1 also activate the expression of the *Period* (Per) and *Cryptochromes* (Cry) gene families. PERs and CRYs protein levels become high during the night, after which they dimerize and translocate to the nucleus to repress CLOCK:BMAL1-mediated transcription. PERs and CRYs undergo a number of post-translational modifications that induce their degradation, required to start off a new circadian cycle. Another loop involves the proteins REV-ERBα/β, whose levels increase during the day and bind specific responsive promoter elements (RRE) and thus inhibit *Bmal1* transcription. At night, REV-ERBα protein amounts are low, allowing *Bmal1* transcription to take place. These transcriptional-translational regulatory loops are operating in most cells and control a remarkable fraction of the mammalian genome.

BMAL1 (Figure 1) (Crane and Young, 2014). They heterodimerize and drive the transcription of a large number of clock-controlled genes (CCGs) by binding to E-box sites within their promoters. CLOCK and BMAL1 also direct the transcription of their own repressors, period (PER) and cryptochrome (CRY) family members, generating a tightly self-regulated feedback loop. During the day, the increase in transcription of *per* and *cry* genes results in the accumulation of the PER and CRY circadian repressors. These, in turn, inhibit CLOCK:BMAL1-driven transcription of *per*, *cry*, and CCGs. The highly controlled degradation of PER and CRY alleviates transcriptional repression and allows CLOCK:BMAL1-mediated transcription to proceed again, establishing cycles in circadian gene expression. Additional levels of circadian regulation exist with the orphan nuclear receptors ROR and REV-ERB that activate and repress transcription of the *Bmal1* gene, respectively. Furthermore, clock proteins are modified in a post-translational manner by phosphorylation, acetylation, ubiquitination, and SUMOylation, adding multiple layers of regulation to the core clock machinery (Crane and Young, 2014; Robles and Mann, 2013).

The CLOCK:BMAL1-driven activation of CCGs deserves special attention in the context of this Review. First, it allows for the circadian regulation of cellular, metabolic, and physiological output functions. Second, transcriptome studies have shown that the overlap of CCGs in different tissues is relatively marginal, questioning the possible contribution of tissue-specific factors to clock control. Finally, it reveals that a large fraction of the genome is potentially under clock control (Masri and Sassone-Corsi, 2010). The intrinsic plasticity of the circadian system could thereby be provided, at least in part, by the potential of expanding or restricting the regulation of CCGs depending on the nutritional, metabolic, and epigenetic state.

The circadian clock is intimately connected to metabolism (Asher and Schibler, 2011; Bass, 2012; Eckel-Mahan and Sassone-Corsi, 2013; Green et al., 2008). Direct evidence is provided by targeted mutations of clock genes in the mouse that yield animals with a variety of metabolic disorders (Sahar and Sassone-Corsi, 2012). From a molecular point of view, it has been shown that the clock machinery controls the expression of essential genes within numerous metabolic pathways. A paradigmatic example is the control by CLOCK:BMAL1 of *Nampt* (nicotinamide phosphorybosil transferase) gene expression (Nakahata et al., 2009; Ramsey et al., 2009). The product of this gene, namely the enzyme NAMPT, functions as the rate-limiting step in the NAD^+-salvage pathway. By controlling *Nampt* cyclic transcription, the clock directs the circadian synthesis of NAD^+ and thereby the potentially cyclic activity of NAD^+-consuming enzymes. This is indeed the case of the NAD-dependent deacetylase SIRT1, an enzyme involved in the control of cellular metabolism, inflammation, and aging (Guarente, 2011). Importantly, SIRT1 had been shown to contribute to CLOCK:BMAL1 function by physically interacting with these circadian regulators (Asher et al., 2008; Nakahata et al., 2008). Recent work has shown that a similar deacetylase, SIRT6, also contributes to circadian control, though acting on a different group of cyclic genes than SIRT1 (Masri et al., 2014). Intriguingly, SIRT6 is chromatin bound, controls lipid metabolism, and is enzymatically activated by fatty acids. SIRT1 and SIRT6 partition the circadian epigenome, leading to segregated control of cellular metabolism (Figure 2), a finding that could be relevant with respect to different nutritional regimes (Eckel-Mahan et al., 2013). In addition, SIRT3, a mitochondrial NAD^+-dependent deacetylase, has been implicated in circadian control of mitochondrial function (Peek et al., 2013), and PARP-1, an NAD^+-dependent ADP-ribosyltransferase, was shown to participate in the phase entrainment of circadian clocks to feeding (Asher et al., 2010). Thus, changes in food composition/feeding time may lead to differential activation of epigenetic and transcriptional control systems through harnessing specialized enzymatic pathways and circadian metabolic sensors.

"Chrono-Nutrition" from Rodents to Humans

Evidence from Studies in Mice

Several studies in mice indicate that changes in feeding schedule carry clear metabolic implications. When mice are housed in constant bright/dim light, they consume more food during the subjective light phase. These mice exhibit significantly increased body mass and reduced glucose tolerance compared

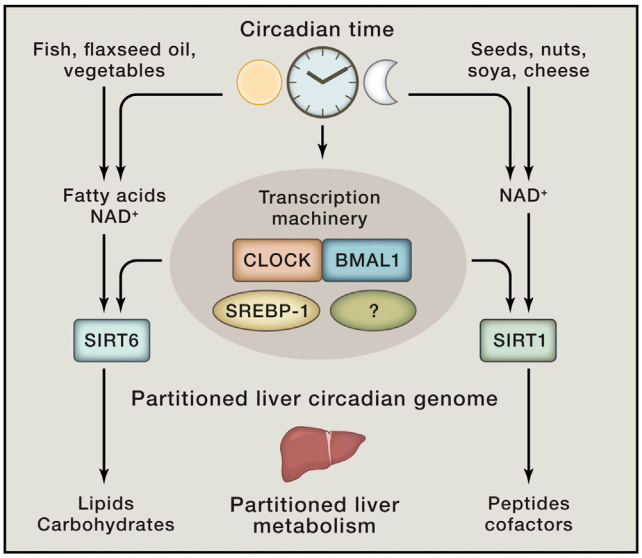

Figure 2. Interplay between Nutrition, the Circadian Clock, and Metabolism

A large body of evidence demonstrates that the circadian clock and cellular metabolism are intimately interconnected. A paradigmatic example is the one represented by two chromatin remodelers, the deacetylases SIRT1 and SIRT6 in the liver (Masri et al., 2014). These two enzymes contribute in partitioning the circadian epigenome as they control distinct groups of genes through different molecular mechanisms, including the activation of alternative transcriptional pathways, such as SREBP-1. The combination of these regulatory events results in segregation of circadian metabolism. SIRT1 participates predominantly in the cyclic control of cofactors and peptides, whereas SIRT6 seems dedicated to cyclic synthesis of lipids and carbohydrates (Masri et al., 2014). As SIRT1 consumes NAD^+ (Guarente, 2011), whereas SIRT6 appears to be activated by free fatty acids (Feldman et al., 2013), these may reflect differential responses to distinct nutritional intakes.

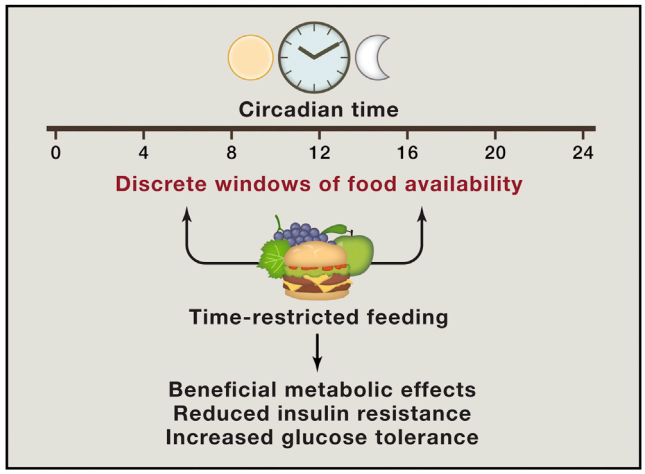

Figure 3. Time-Restricted Feeding and Its Beneficial Effects

Food can be made available only at discrete windows of time within the daily cycle. A large body of evidence indicates that time-restricted feeding is beneficial for a number of metabolic responses, reducing insulin resistance and increasing glucose tolerance (Adamovich et al., 2014b; Hatori et al., 2012; Sherman et al., 2012; Chaix et al., 2014). Although further studies in humans are needed, the beneficial effects of chrono-nutrition should be taken into serious consideration.

with mice under standard light/dark cycles, despite similar caloric intakes and total motor activity (Fonken et al., 2010). Additional support emerges from analysis of genetically modified mouse models. Altered feeding rhythms in these mice further correlated feeding schedule with obesity and metabolic syndrome. In particular, *Clock* mutant mice exhibit greatly attenuated diurnal feeding rhythm. These mice are obese and develop a metabolic syndrome (Turek et al., 2005). Likewise, adipocyte-specific *Bmal1* null mice display increased food intake during the light phase and elevated body weight. Disruption of the clock in the adipocytes seems to modify the circulating concentration of polyunsaturated fatty acids in the hypothalamus, resulting in altered feeding behavior (Paschos et al., 2012).

Time-restricted feeding experiments further highlighted the metabolic effects of feeding schedule. Upon nighttime restricted feeding of regular chow, hepatic triglycerides content in wild-type mice decreases by 50%, whereas the total daily caloric consumption is unaffected (Adamovich et al., 2014b). Similar studies with high-fat diet demonstrated compelling effects on the propensity to develop obesity and metabolic syndrome. Mice under time-restricted high-fat diet consume equivalent calories as those with ad libitum access yet are protected against obesity, hyperinsulinemia, hepatic steatosis, and inflammation (Figure 3). Time-restricted feeding regimen improves CREB, mTOR, and AMPK pathway function and oscillations in expression of circadian core clock and output genes (Hatori et al., 2012; Sherman et al., 2012). It should be noted that both studies applied time-restricted high-fat diet, yet at complete different times throughout the day. The former limited the food availability to 8 hr during the dark phase, whereas, in the latter, food was provided for 4 hr during the light phase. Thus, it is conceivable that the key factor is the time restriction from food per se, rather than its occurrence at a specific circadian time. A recent study from Panda and colleagues further characterized the different requirements for time-restricted feeding to be beneficial (Chaix et al., 2014). Time-restricted feeding appears to be effective against preexisting obesity, there is an after effect upon cessation, and it remains effective even when applied only 5 days per week. This comprehensive study highlighted the effectiveness of time-restricted feeding against different nutritional challenges, including high-fat, high-fructose, and high-fat combined with high-fructose diets, all of which are known to cause dysmetabolism.

Even changes in food availability solely throughout the active phase (e.g., breakfast versus dinner) have been reported to affect body weight. Shibata and co-workers have compared metabolic parameters in mice that consumed only a single large meal at the beginning of the active phase with mice that had an additional relatively small meal at end of the active phase. Although mice in each group consumed an equal amount of food per day, mice on two meals exhibited reduced body weight gain and improved metabolic parameters compared with those on a single meal or freely fed animals (Fuse et al., 2012). Additional studies in rodents demonstrated that early nocturnal fasting increases body weight, whereas late nocturnal fasting reduces weight gain (Wu et al., 2011; Yoshida et al., 2012).

Evidence from Studies in Humans

Although there is a widespread belief that eating late at night carries high risk of developing obesity, the supporting evidence is relatively scarce. In a recent study, the comparison of two isocaloric weight-loss groups revealed greater improvement of metabolic markers in the group given a bigger breakfast and a smaller dinner than vice versa (Jakubowicz et al., 2013). Another study showed that early mealtimes significantly decrease serum lipid levels (Yoshizaki et al., 2013). Moreover, several human epidemiological studies identified a correlation between eating pattern and obesity. For example, breakfast consumption among adolescents was inversely associated with weight gain in a large cohort study (Timlin et al., 2008). Several studies have demonstrated a correlation between short sleep duration (<5 hr) or late sleepers (midpoint of sleep > 5:30 AM) and eating late dinners/consuming more calories late in the evening with significantly higher risk for developing obesity and diabetes (Baron et al., 2011; Hsieh et al., 2011). Moreover, night eating syndrome characterized by a time-delayed eating pattern is positively associated with elevated body mass index (BMI), (Colles et al., 2007). Interestingly, studies both in humans and rats have shown selected food predilections for higher fat composition at dinnertime than at breakfast time (Lax et al., 1998; Westerterp-Plantenga et al., 1996), suggesting a nutritional preference that might be related to obesity associated with late-night feeding. These studies indicate that late dinners carry the risk of obesity in humans; however, they should be cautiously interpreted.

In conclusion, the effects of time-restricted feeding have not yet been thoroughly examined in humans. The current evidence emerging from studies performed in humans is indirect and correlative and calls for additional well-controlled analyses.

Feeding-Fasting Cycles

Feeding time and circadian clocks are tightly intertwined, as feeding schedule has a prominent effect on circadian clocks in peripheral organs. As expected for nocturnal animals, mice mostly consume food during the night. When food is provided exclusively during the day, the phase of peripheral clocks is gradually inverted within several days (Damiola et al., 2000). By contrast, inverted feeding regimen has very little impact on the phase of the master clock in the brain. Therefore, feeding-fasting cycles appear to function as potent timing cues for peripheral clocks, even bypassing the otherwise-dominating synchronization signals emitted by the master clock in the brain. The prominent effect of feeding on circadian rhythmicity is also evident from studies comparing circadian gene expression in mice fed ad libitum with mice under time-restricted feeding regimen. Feeding time had a profound effect on the repertoire, phase, and amplitude of rhythmic gene expression. Specifically, feeding cycles have been shown to rescue the 24 hr rhythmicity in gene expression of numerous transcripts in mice with a genetically disrupted clock (i.e., *Cry1/2* null mice) (Vollmers et al., 2009). Moreover, the diet composition seems to have an impact on the rhythmic feeding behavior in mice and rats (Hariri and Thibault, 2011; Kohsaka et al., 2007). Upon being high-fat fed, mice exhibit altered feeding-fasting cycles very similar to clock-deficient mice (e.g., *Cry1/2* and *Per1/2* double-null mice), as they consume a higher percentage of their daily food intake during the light phase. Concomitantly, circadian gene expression is attenuated (Kohsaka et al., 2007). Interestingly, when mice are fed with high-fat diet exclusively during the night, circadian rhythmicity in gene expression is restored (Hatori et al., 2012).

Molecular Basis for Reprogramming and "Chrono-Nutrition"

Nutritional challenge does not simply disrupt normal circadian rhythmicity. When mice are subjected to high-fat diet ad libitum, the liver clock undergoes a rewiring program that involves a number of molecular mechanisms (Eckel-Mahan et al., 2013). Although many circadian genes lose their cyclic expression due to impaired chromatin recruitment of the CLOCK:BMAL1 activator complex at their promoters, many other genes whose expression is normally non-cyclic become circadian through the cyclic activation of surrogate transcription pathways. These include both PPARγ and SREBP, which begin to activate a large number of target genes by cyclic chromatin recruiting. These findings reveal the remarkable plasticity of the clock system in response to nutritional challenges and indicate that more genes than previously thought have the potential to become circadian, depending on the nutritional, metabolic, and epigenetic state of the cell.

The finding that meal timing has major effects on metabolic and physiological parameters has led to the conviction that, in choosing food, it is not only important to consider its nutritional value but also its timing. So is there an "optimal time" to ingest food? The aforementioned studies delineate the benefits in time-restricted feeding; however, it is still unclear whether there is an "optimal time" for food intake. In some studies, food accessibility was limited to the dark phase, whereas in others, it was limited to the light phase, yet the outcome was very similar. This apparent discrepancy can be resolved by centering on the direct consequences of time restricted feeding. When mice are fed regular chow, they mostly ingest food during the dark phase (Adamovich et al., 2014b). Upon high-fat diet, the situation is aggravated as they consume an almost equal amount of food throughout the day (Hatori et al., 2012). Conceivably, time-restricted feeding generates sharp feeding-fasting cycles, which consolidate circadian rhythmicity in gene expression and circadian activation of various metabolic pathways. This is because clocks in most peripheral organs readily respond to feeding cycles, and feeding time can shift their phase. Upon several days of time-restricted feeding, food availability and the endogenous clocks are aligned, irrespectively on whether the food is provided during the dark or the light phase. Hence, high-fat diet disrupts circadian rhythmicity through dampening of feeding-fasting cycles that serve as an extremely potent zeitgeber for peripheral clocks. It is yet unclear whether this effect of time-restricted feeding by high-fat diet is operating through molecular mechanisms analogous to those involved in the reprogramming of the circadian clock induced by nutritional challenge when food is available ad libitum (Eckel-Mahan et al., 2013).

A Role for the Microbiota

The contribution of the microbiota in regulation of physiology is vast and complex (Henao-Mejia et al., 2013; Tremaroli and Bäckhed, 2012). The impact of the microbiota is determined,

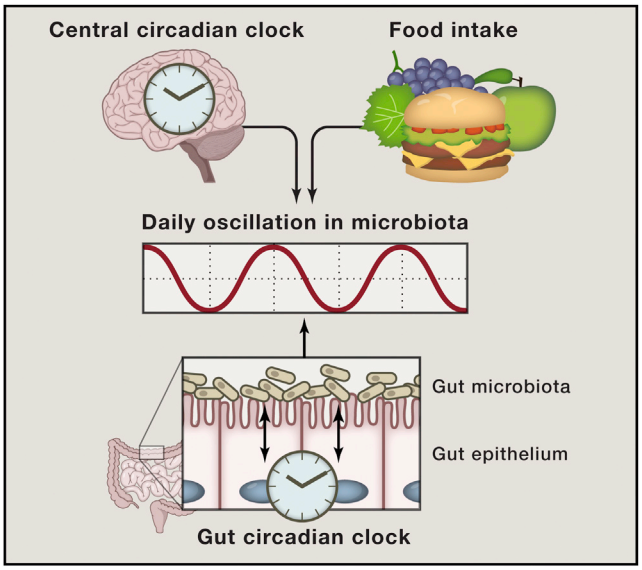

Figure 4. Interplay between the Microbiota and the Gut Circadian Clock

Intestinal cells contain a powerful circadian clock that is connected through yet-ill-defined physiological pathways to the central clock in the SCN. Nutrition is also cyclic, and its processing occurs through the rhythmic metabolism of the gut clock. Here, microbiota have been shown to participate in food processing and to interact with the intestinal clock. This interplay goes both ways, as the gut clock needs the microbiota to function properly, and the microbiota levels oscillate following the gut cycles (Mukherji et al., 2013; Thaiss et al., 2014; Voigt et al., 2014; Zarrinpar et al., 2014). Thus, the circadian clock is a critical component in the relationship between food and the gut.

among various factors, by its diverse composition that includes bacteria, archaea, and fungi. These populate a remarkable variety of sites in the organism of their multicellular hosts (Lozupone et al., 2012). In these locations, they contribute to local organ homeostasis, as well as to system-wide physiology through a number of specialized metabolic and signaling pathways (Lee and Hase, 2014; Sharon et al., 2014).

A large fraction of the microbiota is located in the gastrointestinal tract, a site that also has a powerful circadian clock (Bellet et al., 2013; Hussain and Pan, 2012). The clock in the gut has been shown to participate in the daily cycles of food digestion (Hussain, 2014; Tahara and Shibata, 2013), as well as in coping with the pathogenic condition of *Salmonella* infection (Bellet et al., 2013) and regulation of interleukin-17-producing $CD4^+$ T helper (T_H17) cells that protect against bacterial and fungal infections at mucosal surfaces (Yu et al., 2013). As the gut microbial population regulates the energy derived from food and modulates the levels of host- and diet-derived products, the question of how the gut clock interplays with metabolic pathways and the microbiota is critical (Sharon et al., 2014).

Is the microbiota controlling the gut clock or vice versa? The first answer to this question came from Chambon and colleagues through the analysis of the large intestine, which has the highest concentration of microbiota in mammals (Mukherji et al., 2013). The circadian clock within intestinal epithelial cells (IECs) is responsible for cyclic glucocorticoid production and is under endocrine control from the pituitary-adrenal axis. Remarkably, the IECs clock is profoundly disrupted by depletion of the microbiota, leading to altered corticosteroids levels and consequent metabolic disorders. Thus, the microbiota determines the appropriate function of the gut clock, most likely contributing to additional, system-wide cyclic homeostasis. This control operates through the innate immune receptors TLRs and NOD2, whose expression in IECs is cyclic and under clock regulation (Mukherji et al., 2013; Silver et al., 2012). Thus, changes in the microbiota levels and composition induced by different types of nutritional regimens could differentially regulate the gut clock and thereby influence the organism's homeostasis. This is indeed the case. The composition of the microbiota undergoes diurnal oscillations in both mice and humans (Thaiss et al., 2014), and these oscillations are disturbed in mice with a genetic mutation in the clock system, as well as in jet-lag experiments (Thaiss et al., 2014; Voigt et al., 2014). It appears thereby that the relationship between the gut epithelium of the host and the microbiota goes both ways, where both cell populations influence each other's physiology (Figure 4).

Another key component in the equation is food, whose intake is also cyclic, following an intricate system of neuronal and endocrine control. Different nutritional challenges alter the composition of the microbiota, and specific members of the intestinal microbiota have been linked with metabolic disease (Zarrinpar et al., 2014). Yet, how does time-specific and diet-specific food intake alter in parallel the gut clock and the microbiota? High-throughput genomic and metabolomics analyses have revealed that specific "signatures" exist for each dietary condition, as well as for time-restricted feeding (Zarrinpar et al., 2014). These intriguing results reveal the complexity of the gut homeostasis, as well as the importance that key molecular pathways, such as TLRs and NOD2, must play. It remains to be established the role of the clock in IECs cells by using tissue-specific mutations of clock proteins in the mouse, as well as the critical effect that the circadian gut-microbiota interplay most likely has on other metabolic organs, such as the liver, fat, and muscle. Finally, the fascinating influence that the microbiota appears to have on neuronal functions (Mayer et al., 2014) begs the question of whether the central clock or other brain structures may be receiving "nutritional" information in a cyclic manner through the gut-microbiota interplay.

Circadian Metabolomics

In the last decade, numerous transcriptome profilings performed throughout the day in various peripheral organs have highlighted the pervasive circadian control of metabolism and physiology (Masri and Sassone-Corsi, 2010; Green et al., 2008). Circadian transcriptomes are considered a hallmark for circadian rhythmicity and shed light on metabolic pathways that are potentially under circadian control. In recent years, in view of the expansion of cutting-edge technologies, several research groups commenced employing high-throughput metabolomics approaches to study circadian rhythms. These recent advances evinced multiple layers of regulation and complexity in circadian control. Metabolomics enables the systematic study of the unique chemical fingerprints involved in biological processes. This technology instigated the progress from learning individual compounds to exploring a broad combination of well-defined

metabolites and even to the identification of novel, previously uncharacterized metabolites. Early studies by Ueda and coworkers established a reliable metabolite timetable method to determine internal body time by quantifying the spectra of hundreds of metabolites throughout the day both in mice (Minami et al., 2009) and human blood samples (Kasukawa et al., 2012). Hence, similar to transcriptomes, metabolomes can be employed as a signature for endogenous time.

Subsequent studies were designed to identify metabolic pathways that exhibit daily oscillations and to dissect their circadian control. Metabolomics study of plasma and saliva samples from humans revealed that ~15% of all identified metabolites oscillate in a circadian manner, independently of scheduled sleep and food intake (Dallmann et al., 2012). Notably, a high proportion of rhythmic metabolites in blood plasma were fatty acids. Likewise, daily oscillations in blood plasma metabolites have been observed in human individuals under normal conditions (Ang et al., 2012). Similar studies conducted with mouse liver samples from wild-type and clock mutant mice identified clock-controlled circadian oscillation of various groups of metabolites, including lipids and, more specifically, fatty acids (Eckel-Mahan et al., 2012). Circadian metabolomics, therefore, do not only serve as a reliable readout for internal time but also constitute a productive tool to study the interplay between clocks and metabolism.

Circadian Lipidomics

Lipid homeostasis appears to be under circadian control, and disruption of circadian rhythmicity is associated with dyslipidemia and obesity in various clock mutant mouse models (Gooley and Chua, 2014; Sahar and Sassone-Corsi, 2012). These include elevated VLDL triglyceride levels in *Rev-Erbα* null mice (Raspé et al., 2002); dampening of triglycerides oscillations in blood plasma of *Bmal1*$^{-/-}$ mice (Rudic et al., 2004); hyperlipidemia and hepatic steatosis in *Clock* mutant mice (Turek et al., 2005); and reduced whole-body fat, total triglycerides, and fatty acids in blood plasma of *Per2*$^{-/-}$ mice (Grimaldi et al., 2010). Concomitantly, lipids also appear to play a role in circadian control (Adamovich et al., 2014a). Lipidomics analysis on human blood plasma revealed that ~13% of lipid species exhibited circadian oscillations (Chua et al., 2013), with high prevalence of diglycerides, and triglycerides that peaked around the circadian time (CT) 8 (i.e., 8 hr after lights are turned on). This is in line with the phase observed for lipid accumulation in previous human plasma metabolomics (Dallmann et al., 2012). Daily changes in triglycerides were also observed in blood plasma of rats—total plasma triglyceride levels were elevated during the night (CT 18) (Pan and Hussain, 2007). Thus, triglyceride levels in blood plasma reach their zenith levels during the active phase, namely in human during the day and in rodents during the night.

A recent comprehensive circadian lipidomics analysis of mouse liver has identified that ~17% of quantified lipids display circadian rhythmicity (Adamovich et al., 2014b). Notably, the majority of the oscillating lipid species were triglycerides (~33%) and reached their peak levels in the liver during the subjective light phase (i.e., CT8). The findings that triglycerides accumulate in rodent plasma during the active phase and in liver during the rest phase may suggest that triglyceride levels build up in different phases in liver and blood in a manner that most likely depends on feeding-fasting cycles and circadian clocks. Because food is a major source for triglycerides, it is conceivable that these accumulate first in the blood upon food ingestion during the active phase and are subsequently deposited in peripheral organs such as the liver during the rest phase. Surprisingly, a similar fraction of lipids (~17%) was oscillating in both wild-type and clock-disrupted mice (i.e., *Per1/2* null), most notably triglycerides. However, they largely differed in their accumulation phase and composition (Adamovich et al., 2014b). These observations are intriguing, as mice lacking both PER1 and PER2 are behaviorally arrhythmic under constant darkness, and their circadian expression of core clock genes is largely abolished. Further studies should aim at identifying the molecular mechanisms that drive the circadian accumulation of triglyceride in the absence of a functional clock feedback loop.

Integrative Omics Analysis

The above-described studies emphasize the need for an integrative and comprehensive exploration of the data emerging from the different omics. Indeed, a database (CircadiOmics database; http://circadiomics.igb.uci.edu/) that integrates circadian genomics, transcriptomics, proteomics, and metabolomics has been generated (Eckel-Mahan et al., 2012; Patel et al., 2012). It facilitates the use of the current data for deciphering circadian control of various metabolic pathways. It also illustrates the coherence that specific metabolic pathways share with distinct circadian transcription nodes. More importantly, these studies shed some light regarding the complexity of circadian control. First, in contrast to the high-amplitude oscillations in transcript abundance derived from numerous transcriptome studies, daily changes in protein and metabolite levels appear to be significantly shallower. For example, metabolite oscillations in human blood samples ranged around 2-fold (Ang et al., 2012; Dallmann et al., 2012), and most lipid species in the liver oscillated with an amplitude of ~1.5-fold (Adamovich et al., 2014b). A similar trend was observed with circadian proteomics data sets in which, for most cases, the amplitude of protein oscillations is relatively shallow (Mauvoisin et al., 2014; Masri et al., 2013; Reddy et al., 2006; Robles et al., 2014). Second, comparison of circadian transcriptome, proteome, and metabolome data demonstrated that, in some cases, they do not overlap. For instance, the levels of many proteins encoded by rhythmically expressed mRNAs do not oscillate at significant levels, whereas some proteins produced by constantly expressed transcripts do cycle in abundance. Third, in contrast to the disparate expression phases of genes encoding enzymes that participate in hepatic triglyceride homeostasis, triglyceride accumulation in the liver is highly coordinated and peaks toward the end of the light phase (Adamovich et al., 2014b). The discrepancy between the phase and amplitude of oscillations in transcript levels in comparison with proteins and metabolites probably reflects the complexity involved in their accumulation. Specifically, control of metabolites concentration often requires multiple steps and relies on the activity of several enzymes, substrates, and cofactor availability.

Concluding Remarks

During the past decade, remarkable progress has been made in our understanding of the mammalian circadian timing system.

The exciting emerging era of proteomics, metabolomics/lipidomics, commence to deepen our understanding of circadian rhythms.

Several circadian transcriptome studies have been conducted in the last decade characterizing circadian gene expressing in different organs, mouse strains, and feeding conditions (as an example, see Zhang et al., 2014). By contrast, circadian metabolomics are still in their infancy and should be cautiously analyzed and interpreted. Indeed, as aforementioned, the amplitude of metabolites oscillations is often relatively shallow and, hence, more likely to be affected by the experimental design and quantification methods. Future studies are expected to shed more light and provide better tools to tackle these caveats. In any rate, these approaches promote the identification of pathways that couple the molecular clock to metabolism, uncovering new circadian outputs and dissecting the interplay between environmental cues (e.g., feeding) and clocks in circadian control. Concomitantly, the flare-up in nutritional studies that address the effect of feeding schedule on obesity and metabolic syndrome, together with the entry of new players such as microbiota into the circadian playground, reveals the true potential of operating on circadian clocks as strategy toward therapeutics for metabolic diseases and other pathologies.

ACKNOWLEDGMENTS

Due to space limitations, we were, unfortunately, unable to comment on all of the relevant studies and thus apologize for not citing all pertinent references. Work in the laboratory of G.A. was supported by the Israel Science Foundation (ISF 138/12), the Abish-Frenkel Foundation, the HFSP Career Development Award (HFSP CDA00014/2012), and the European Research Council (ERC-2011 METACYCLES 310320). Work in the laboratory of P.S.-C. is supported by the NIH, ABOCA SpA (Italy), Institut National de la Sante et Recherche Medicale (INSERM, France), and a Novo Nordisk Foundation Challenge Grant.

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Cell

I'm Eating for Two: Parental Dietary Effects on Offspring Metabolism

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http://dx.doi.org/10.1016/j.cell.2015.02.021

It has long been understood that the pathogenesis of complex diseases such as diabetes includes both genetic and environmental components. More recently, it has become clear that not only does an individual's environment influence their own metabolism, but in some cases, the environment experienced by their parents may also contribute to their risk of metabolic disease. Here, we review the evidence that parental diet influences metabolic phenotype in offspring in mammals and provide a current survey of our mechanistic understanding of these effects.

Introduction

Metabolic diseases contribute a massive burden to healthcare throughout the world. Although a large number of Mendelian disorders of metabolism have been identified, the vast majority of metabolic disease burden stems from complex diseases such as diabetes, which have both heritable genetic components, as well as contributions from a patient's lifestyle and environmental exposures. In recent years, genome-wide association studies (GWAS) have uncovered a large number of sequence variants that significantly contribute to the overall heritability of metabolic diseases or to various morphological traits such as body mass index (BMI) or adiposity (Travers and McCarthy, 2011). However, an emerging theme from GWAS is that all genetic variants identified typically explain a small fraction of the heritability of a given complex trait such as diabetes. As one of many examples, in human populations of European descent, all significant GWAS "hits" together explain ~10% of the heritability of type 2 diabetes and ~5% of heritability of fasting plasma glucose (Bonnefond et al., 2010; Morris et al., 2012; Scott et al., 2012). In general, a number of factors could explain this so-called "missing heritability," including many rare variants contributing to a given phenotype, and epistasis. In addition to these, it is increasingly appreciated that epigenetics—the inheritance of information beyond DNA sequence—could potentially contribute to the heritability of such diseases. Indeed, a substantial body of evidence links parental nutritional status to metabolic traits in offspring, possibly providing an explanation for a subset of missing heritability in metabolic diseases.

For example, the development of disease later in life has been linked to exposure to an adverse intrauterine environment, as observed in offspring of pregnancies complicated by intrauterine growth restriction (IUGR), obesity, or diabetes (Hales and Barker, 1992, 2001; Kermack et al., 1934; Ravelli et al., 1976, 1998; Valdez et al., 1994). The period from conception to birth is a time of rapid growth, cellular replication and differentiation, and functional maturation of organ systems. These processes are very sensitive to alterations in nutrient availability, and an abnormal intrauterine metabolic milieu can thus have long-lasting effects on the offspring. Perhaps the clearest case of how nutrient availability during pregnancy affects long-term health and disease in the offspring is exemplified by the Dutch Hunger Winter. This period of famine occurred in the western part of the Netherlands during the winter of 1944–45; the period of famine was clearly defined, and official food rations were documented. Extensive health care and birth weight registries still exist for this population, and numerous studies on this cohort have clearly shown that prenatal exposure to famine is associated with the later development of diseases such as obesity, diabetes, and cardiovascular disease (Lumey et al., 2007).

David Barker and Nicholas Hales coined the term "fetal origins of adult disease" based on their studies demonstrating a relationship between low birth weight and the later development of cardiovascular disease and impaired glucose tolerance (Hales and Barker, 1992, 2001). This concept has been broadened to include nutritional excess and/or diabetes during pregnancy. Multiple studies in diverse populations throughout the world have demonstrated a significant correlation between low birth weight, maternal obesity, or maternal diabetes and the later development of chronic diseases such as type 2 diabetes and or obesity (reviewed in Duque-Guimarães and Ozanne [2013] and Simmons [2011]). It must be acknowledged that these epidemiology studies are cross-sectional (rather than longitudinal) and, with the exception of the study by Rich-Edward and colleagues (Rich-Edwards et al., 1999), typically have small numbers of subjects. Nonetheless, the human data are compelling in aggregate and are well supported by animal studies in multiple species, including non-human primates.

Although most studies have concentrated on the maternal environment, it is also becoming increasingly evident that paternal exposures can result in the later development of metabolic disorders in the offspring (Rando, 2012). Further, both human and animal studies demonstrate intergenerational transmission of the maternal or paternal phenotype (see F2

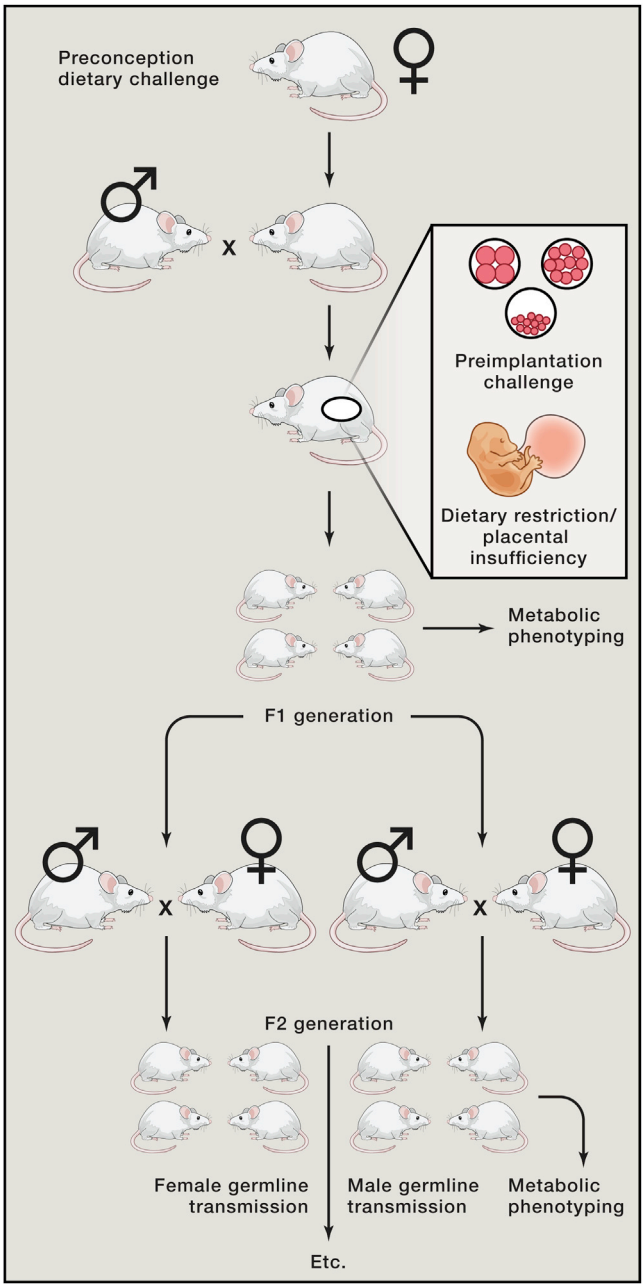

Figure 1. Schematic for Maternal Effect Paradigms

Maternal effect paradigms typically involve altered access to nutrients, with key paradigms including under- or overnutrition prior to conception, during preimplantation development, or later in pregnancy. Phenotypes are then typically studied in F1 offspring. In a subset of experimental systems, F1 offspring are bred either with control animals or with one another to identify multigenerational effects of fetal undernutrition.

effects in Figure 1), suggesting the possibility that an epigenetic mechanism is mediating these effects (see below). In this Review, we will highlight the most relevant animal models and molecular mechanisms underlying maternal and paternal transmission of information about nutritional status to offspring.

The Maternal Environment

Decreased Nutrient Availability: Fetal Growth Restriction

A number of animal models have been used to assess the role of gestational environmental effects in inducing chronic diseases in the offspring (reviewed in Fowden and Forhead [2004] and McMillen and Robinson [2005]). In such models, pregnant females are subject to a variety of challenges, including (1) caloric or protein restriction, (2) glucocorticoid administration, and (3) induction of uteroplacental insufficiency via methods such as ligation of the uterine artery. Although the outcomes observed in offspring differ in detail depending on the organism studied and the details of the challenge, a large number of related studies all find that severe maternal undernutrition during gestation can have an impact on offspring glucose metabolism that persists into adulthood. For instance, in the rat, maternal protein restriction (by ~40%–50% of normal intake) during gestation and lactation can impair insulin sensitivity, induce β cell defects, and cause hypertension in offspring (Dahri et al., 1991), and as these offspring age, they develop hyperglycemia characterized by defects in insulin signaling in muscle, adipocytes, and liver (Fernandez-Twinn et al., 2005; Ozanne et al., 2003; Petry et al., 2001). Beyond the lasting effects of gestational environment on the F1 generation (Figure 1), some reports find that fetal undernutrition can alter phenotypes even in the F2 generation (Jimenez-Chillaron et al., 2009; Radford et al., 2012; Radford et al., 2014), a result that will be discussed further in the paternal effects section.

Mothers can buffer the effects of various dietary regimens on fetal access to nutrients—only in the face of severe maternal malnutrition is fetal growth adversely affected by maternal diet. As a result, IUGR in humans seldom occurs as a result of maternal undernutrition and instead most often is a consequence of uteroplacental insufficiency. Conditions such as maternal hypertension, pre-eclampsia, anemia, smoking, and poor placentation are common causes of uteroplacental insufficiency. To model uteroplacental insufficiency in the laboratory, bilateral uterine artery ligation at day 18 of gestation in the rat (where term is 22 days) is used to restrict fetal growth. This paradigm recapitulates key aspects of human IUGR, with reduced levels of glucose, insulin, insulin-like growth factor 1 (IGF-I), amino acids, fatty acids, and oxygen being available to the fetus (Simmons et al., 1992, 2001). In adulthood, IUGR rats develop diabetes with a phenotype similar to that observed in the human with type 2 diabetes: progressive dysfunction in insulin secretion and insulin action (Simmons et al., 2001; Stoffers et al., 2003).

These and many other studies provide a clear link between nutrient availability during gestation and future metabolic phenotype in offspring. A burgeoning field seeks to understand how a fetus' early environment can alter the metabolism of the offspring long after access to adequate nutrition has been restored.

Proximate Cellular Mechanisms: Mitochondrial Dysfunction and Oxidative Stress. How does a resource-poor environment affect developing organ systems? Although many metabolic pathways and signaling systems are of course impacted by levels of hormones and available nutrients, it appears that reprogramming of mitochondrial function represents one of the

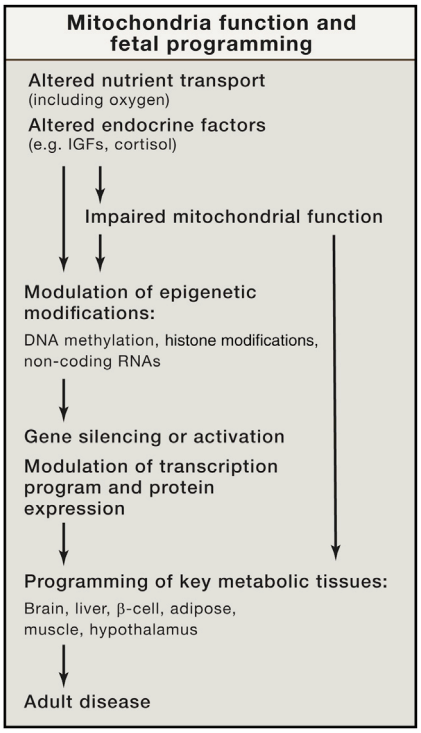

Figure 2. Mechanisms Linking Fetal Nutrient Supply to Later Phenotypes

Uteroplacental insufficiency decreases availability of key substrates such as oxygen and glucose to the fetus. Altered substrate availability (e.g., decreased acetyl Co-A or S-Adenosyl methionine) can directly influence epigenetic mediators, resulting in epigenetic modifications of key genes. Decreased levels of glucose and oxygen can also impair mitochondrial function and increase production of ROS. These processes can have a detrimental effect on numerous cellular pathways, culminating in fetal programming.

key adaptations enabling the fetus to survive in a limited nutrient environment (Pejznochova et al., 2010; Sakai et al., 2013). In response to a reduction in energy supply and oxygen secondary to uteroplacental insufficiency, mitochondria are activated in the fetus to satisfy the cellular need for energy (Chang et al., 2013; Lattuada et al., 2008). However, activated mitochondria can lead to increased production of reactive oxygen species (ROS) and to oxidative stress (Figure 2), which can have deleterious effects in cells that have a high energy requirement, such as the pancreatic β cell (Newgard and McGarry, 1995; Simmons, 2005). Consistent with this, uteroplacental insufficiency has been shown to induce oxidative stress and marked mitochondrial dysfunction in the β cells, hepatocytes, myocytes, and placenta of IUGR offspring (Myatt, 2010; Peterside et al., 2003; Selak et al., 2003; Simmons et al., 2005). Islets of IUGR offspring exhibit progressive declines in ATP production and activities of complexes I and III of the electron transport chain, as well as accumulating mutations and decreasing content of mitochondrial DNA with age (Simmons et al., 2005).

Although β cells are particularly susceptible to oxidative stress, mitochondrial dysfunction is not limited to the β cell in the IUGR animal. Oxidation rates of pyruvate, glutamate, succinate, and α-ketoglutarate are markedly decreased in isolated hepatic mitochondria from IUGR pups (prior to the onset of diabetes), and this derangement predisposes the IUGR rat to increased hepatic glucose production by suppressing pyruvate oxidation and increasing gluconeogenesis (Park et al., 2003; Peterside et al., 2003). Similar defects are observed in IUGR muscle, where impaired ATP synthesis compromises energy-dependent GLUT4 recruitment to the cell surface, glucose transport, and glycogen synthesis, all of which could potentially contribute to the insulin resistance and hyperglycemia of type 2 diabetes (Selak et al., 2003).

Mitochondrial abnormalities have also been observed in other animal models of IUGR. Mitochondrial DNA content is reduced in liver, pancreas, and skeletal muscle of male offspring of rat dams fed a low-protein diet during pregnancy and lactation (Park et al., 2004), and similar findings have been reported in a pig IUGR model (Liu et al., 2012). Finally, more recently, a targeted metabolomics study in a rabbit IUGR model (unilateral uterine artery ligation) revealed a significant increase in metabolites associated with oxidative stress in IUGR rabbit brain (van Vliet et al., 2013). Thus, multiple models of fetal growth restriction in different species show oxidative stress in several tissues, making mitochondrial dysfunction a common feature of fetal growth restriction and a good candidate to play a mechanistic role in the later development of disease in adulthood (Petersen et al., 2004).

Much remains to be learned about the proximal mechanisms by which an altered intrauterine milieu affects developing animals. Intervention studies will be required to definitively identify whether and how mitochondrial alterations contribute to metabolic changes in offspring. Moreover, although altered mitochondrial function is a common outcome of IUGR, it also is clear that many other signaling pathways are activated, often in a tissue-specific manner, in IUGR offspring as they develop. A key challenge for the field will be to understand how these different pathways interact to result in the long-term outcomes observed in IUGR individuals.

Cellular and Molecular Mechanisms: Epigenetic Regulation. The central question in considering the developmental origins of adult disease is how a transient stimulus occurring early in life can give rise to long-lasting phenotypic consequences that persist many cellular generations after termination of the nutritional challenge. Cellular memory systems such as those involved in cell state maintenance—collectively referred to as epigenetic inheritance mechanisms—play widespread roles in maintaining phenotypes across tens or hundreds of mitotic generations following transient developmental cues and thus represent the likeliest candidates underlying persistent effects of the fetal environment later in life.

Intriguingly, the metabolic or nutritional state of the organism directly influences epigenetic modifications, as essentially all known epigenetic modifications rely upon substrates derived from intermediary metabolism such as S-adenosyl methionine (SAM), acetyl CoA, α-ketoglutarate, and nicotinamide adenine dinucleotide (NAD^+) (Kaelin and McKnight, 2013). A role for parental nutrition in regulation of DNA methylation in offspring is best exemplified by experiments performed in *agouti viable yellow* (A^{vy}) mice or *axin fused* ($Axin^{Fu}$) mice (reviewed in Martin et al., 2008). A^{vy} mice carry an intracisternal A particle (IAP)

retrotransposon upstream of the *Agouti* gene, and these animals exhibit a range of coat colors that is linked to repression of *Agouti* transcription in some animals by encroaching cytosine methylation from the IAP element. When pregnant agouti-colored female A^{vy} mice are fed a diet supplemented with methyl donors, a larger percentage of offspring have a wild-type coat color compared to offspring of mothers fed standard chow. These phenotypic changes are associated with changes in DNA methylation at the IAP element (Cooney et al., 2002; Dolinoy et al., 2006; Waterland et al., 2006), suggesting that early-life access to specific metabolites can stably change gene expression via epigenetic modifications, thus affecting the phenotype of the adult.

A number of animal studies have shown that maternal nutritional status and fetal nutrient availability induce epigenetic modifications across the genome—genome-wide DNA hypomethylation, as well as an increase in total histone H3 acetylation, is observed in postnatal IUGR liver (MacLennan et al., 2004). Beyond such global effects of IUGR, focal changes in epigenetic marks have also been described at specific target genes, as for example the promoters of *Ppargc1* and *Cpt1* exhibit local increases in H3 acetylation even relative to the increased background H3 acetylation observed in neonatal IUGR liver (Fu et al., 2004). Among these locus-specific changes, promoters of key developmental transcription factors have been the subject of the greatest interest. Most notably, in the rat model, fetal growth restriction induces epigenetic modifications that alter the expression of the homeodomain-containing transcription factor PDX1 (Park et al., 2008). PDX1 plays a critical role in the early development of both endocrine and exocrine pancreas and then in the later differentiation and function of the β cell, making regulation of this target a key focus for understanding the pathological outcomes of IUGR. Levels of *Pdx1* mRNA are reduced by more than 50% in IUGR fetal rats as early as 24 hr after the onset of growth retardation, and altered *Pdx*1 expression persists after birth. Repression of *Pdx1* occurs in two waves, as early repression of this gene involves histone deacetylation by the mSin3A complex, followed later by H3K9 dimethylation and eventual recruitment of DNMT3A and cytosine methylation. Prior to cytosine methylation—at the neonatal stage—this epigenetic process is reversible and may define an important developmental window for therapeutic approaches. Indeed, hormone treatments that result in early reversal of *Pdx1* deacetylation also prevent the onset of diabetes in the IUGR rat (Pinney et al., 2011), although whether epigenetic misregulation of *Pdx1* is responsible for metabolic phenotypes in offspring remains to be directly tested. Intriguingly, *Pdx1* expression is also markedly decreased, with correspondingly increased methylation of a key enhancer element, in islets of humans with type 2 diabetes (Yang et al., 2012), further emphasizing this locus as a target of significant therapeutic interest in humans.

Pdx1 represents one of several well-characterized target loci that suffer long-term epigenetic reprogramming in response to maternal dietary conditions. As another example, the pancreatic transcription factor Hnf4α is also epigenetically regulated by maternal diet in rat islets from offspring of protein-restricted dams (Sandovici et al., 2011). Here, increased levels of DNA methylation and repressive histone modifications at the P2 promoter of *Hnf4α* were linked to a significant reduction in expression, while reversal of DNA methylation and histone modifications could re-activate transcription of *Hnf4α* via the P2 promoter. Another relatively well-characterized epigenetic target of caloric restriction during pregnancy and lactation is the *Glut4* promoter in skeletal muscle, where diminished histone acetylation and increased H3K9 methylation occurs in response to maternal caloric restriction, although in this case, there is no apparent increase in cytosine methylation (Raychaudhuri et al., 2008). These events effectively create a metabolic knockdown of *glut4*, an important regulator of peripheral glucose transport and insulin resistance. Taken together, these studies show that histone modifications can be stably altered at specific genomic loci in response to a calorie-restricted model of IUGR.

These and ongoing studies identify epigenetic changes associated with pregestational access to nutrients, thus providing compelling hypotheses for the mechanism by which early environment exerts long lasting phenotypic effects (Figure 2). Two outstanding questions are raised by such findings. First, the signaling mechanisms responsible for establishing or altering epigenetic marks at specific target genes largely remain obscure. Does fetal undernutrition affect *Pdx1* histone acetylation by altering global levels of Acetyl-CoA, with genomic variability in activity of specific histone acetyltransferases at individual genes making specific genes more or less responsive to Acetyl-CoA changes in a given concentration window? Or, presumably more likely, are histone-modifying enzymes specifically targeted to individual target genes via signal-induced recruitment by sequence-specific DNA-binding proteins? Second, understanding the epigenetic marks that play the greatest role in contributing to eventual phenotypic outcomes will require directed epigenetic interventions. For example, inducible CRISPR-targeted recruitment of histone acetylases to the *Pdx1* promoter could be used to assess the importance of IUGR-driven deacetylation of this locus in the pathogenesis of β cell dysfunction in offspring. Both of these areas of research should yield great insight over the coming decade.

Increased Nutrient Availability: Obesity in Pregnancy

Although it has long been understood that inadequate nutrition during pregnancy can have lasting metabolic effects on children, only more recently has the converse situation of maternal overnutrition been appreciated as a contributor to adult disease. Obesity is a growing threat worldwide, and its prevalence has risen dramatically over the last two decades, with many studies indicating that early life exposures are important in promoting adult obesity. There are a number of critical periods during childhood that appear to influence the later development of obesity, including early infancy, 5–7 years of age (known as the adiposity rebound period), and puberty (reviewed in Dietz, 2004). It is becoming increasingly evident that the prenatal stage also represents a window of susceptibility for early life exposures (Bayol et al., 2005; Chang et al., 2008; Guo and Jen, 1995; Jungheim and Moley, 2010; Simmons, 2005; Sullivan et al., 2011), as offspring of obese humans and animals exhibit increased fat mass very early in life. In fact, several studies suggest that obesity can also influence molecular aspects of the oocyte and early embryo (Jungheim and Moley, 2010; Jungheim et al., 2010; Marquard et al., 2011), raising the possibility that exposure

to an adverse metabolic milieu even prior to pregnancy (Figure 1) could account for some of the metabolic outcomes observed in offspring. Indeed, by carrying out reciprocal two-cell embryo transfers between obese mice or lean mice, we have separated the effects of obesity on the oocyte from its effects on the gestational environment. Pre-gestational exposure to maternal obesity impaired fetal and placental growth despite the conceptus being exposed to a normal gestational milieu after transfer, with changes in placental gene expression being observed for offspring generated from high-fat oocyte donors (Sasson et al., 2015). Alterations are also observed in the brain reward system of offspring generated using this paradigm (Grissom et al., 2014), suggesting that obesity prior to pregnancy may program food preferences and/or intake. These results have profound implications, as it is possible that the effects of maternal obesity may be thus transmitted to subsequent generations.

Cellular Mechanisms: Inflammation and Oxidative Stress. Obesity, in both the non-pregnant and pregnant state, has long been understood to involve increased inflammation and oxidative stress in a multitude of tissues throughout the body. Release of inflammatory molecules occurs not only in metabolic and immune tissues, as the placenta can also contribute to the inflammatory/oxidant state; a number of studies have reported that expression of cytokines, inflammation-related genes, and genes linked to oxidative stress are markedly elevated in placenta of obese women (Hauguel-de Mouzon and Guerre-Millo, 2006; Roberts et al., 2011; Taylor and Poston, 2007; Zaretsky et al., 2004; Zhu et al., 2010). Data from animal studies suggest that oxidative stress is directly linked to the development of obesity in offspring. In rats, exposure to maternal obesity prior to and during pregnancy leads to mild oxidative stress even prior to implantation, and this altered state persists into early life (Sen and Simmons, 2010). Importantly, administration of an antioxidant supplement to the dam completely prevents the development of adiposity and glucose intolerance in the offspring, providing evidence for a causal role for oxidative stress (in the mother or in the embryo) in the later development of obesity.

A key question remains to be addressed: how does inflammation and oxidative stress result in increased accumulation of fat? Multiple studies have shown that inflammatory mediators and oxidants enhance adipogenesis, promote differentiation of adipocyte progenitors, and increase lipid deposition in the adipocyte (reviewed in Ortega and Fernández-Real, 2013). However, it remains to be determined whether or not these pathways are operative in the early embryo or fetus. It is also well known that a normal redox state plays a fundamental role in embryonic development, and it is possible that a more oxidizing environment alters lineage specification. Consistent with this notion is the finding that expression of a number of genes regulating adipocyte commitment is altered in blastocysts of obese animals (Sen and Simmons, 2010). As in the case of IUGR, a multitude of signaling cytokines and metabolites differ in abundance between obese versus non-obese pregnancies, and much work remains to be done to define the contributions of individual signaling pathways to phenotypic outcomes in the offspring.

Cellular and Molecular Mechanisms: Epigenetic Regulation of Adipogenesis. The observation that increased fat mass in offspring of obese animals occurs very early in life suggests that adipocyte development per se may play an important role in the genesis of obesity in the offspring. This is not to say that increased maternal adiposity does not program appetite or energy expenditure later in life—it likely does (Chang et al., 2008; Grissom et al., 2014). However, several lines of evidence also implicate potentiation of adipogenesis in early life as a causal mechanism for the later development of obesity.

Adipocyte precursor cells isolated from fat express high levels of mesenchymal stem cell markers such as *Pref-1*, *Wisp2*, and anti-angiogenic factors, which together maintain the adipocyte precursor cell in a committed but undifferentiated state (Rodeheffer et al., 2008; Wagner et al., 2005). Obesity in pregnancy significantly increases expression of similar genes in fat tissue of young offspring, and this altered expression persists in fat tissue of older offspring of obese dams, suggesting the possibility that continuing expansion of the adipocyte precursor pool may help explain the progressive increase in fat mass in the offspring (Sen and Simmons, 2010). Further support for this concept was recently shown in studies by Du and colleagues (Yang et al., 2013), who find that expression of Zfp423, a key transcription factor committing cells to the adipogenic lineage, is significantly increased in e14.5 embryos of obese dams. Levels of many other key adipogenic, as well as lipogenic, regulators (Zfp423, PPARγ, C/EBPα, C/EBP-β, SREBP-1, FASN, SCD-2, and ELOVL-6) are markedly increased in white adipose tissue of offspring of obese dams (Borengasser et al., 2014). Notably, many of these expression changes in offspring are accompanied by local changes in cytosine methylation, again providing a plausible mechanism by which early fetal experience can induce persistent phenotypic changes in offspring. How the nutritional environment signals to these genes and whether these epigenetic marks are responsible for long-term phenotypic outcomes remain to be studied.

Epigenetic Epidemiology

The findings described above linking over- or under-nutrition during pregnancy to epigenetic changes in offspring naturally raise the opportunity for epigenomic surveys in humans to potentially identify targets for therapeutic intervention. Indeed, there are numerous studies in humans examining the relationship between fetal nutrient availability and epigenetic modifications in the offspring (Rakyan et al., 2011). At present, many of these are confounded by small sample size, cellular heterogeneity of tissues examined, and lack of validation. For example, most DNA methylation assays are performed in total peripheral blood monocytes, where the unique methylation profiles of the various cellular lineages complicate interpretation of the data. Despite these issues, multiple studies in diverse populations report changes in DNA methylation associated with low birth weight and or altered nutrient availability. Thus, it is likely that an adverse in utero milieu does indeed induce epigenetic modifications in the offspring, but whether these modifications have biological relevance remains to be determined. The field of ''epigenetic epidemiology'' remains an active and growing field of investigation, and we anticipate exciting advances in this area in the coming years.

Multigenerational Transmission: Maternal

That the in utero environment influences phenotypes in the offspring is not particularly surprising, given that the offspring

directly experience the gestational environment. However, it is also becoming increasingly evident that the effects of an altered in utero milieu may be even transmitted to subsequent generations that did not experience the environment (reviewed in Aiken and Ozanne, 2014). For example, in humans, studies done in a Swedish population showed that overnutrition in the grandparents during early childhood is associated with increased risk of cardiovascular disease in grandchildren (Kaati et al., 2002; Pembrey et al., 2006). Further, obesity in the grandchild is linked to obesity of the grandparent independent of parental weight (Davis et al., 2008). The transmission of programmed effects to subsequent generations has been reported in a number of animal models, including prenatal glucocorticoid overexposure in rats (this model has clinical relevance as glucocorticoids are commonly given to women threatening preterm delivery) (Drake et al., 2005, 2011), maternal undernutrition in mice and rats (Blondeau et al., 2002; Harrison and Langley-Evans, 2009; Jimenez-Chillaron et al., 2009; Thamotharan et al., 2007), neonatal overnutrition in the mouse induced by a reduction in litter size (Pentinat et al., 2010), and maternal obesity in mice (Dunn and Bale, 2009, 2011; Gniuli et al., 2008). The majority of these studies end at the F2 generation, with relatively few studies having been carried out in the F3 generation and beyond (Dunn and Bale, 2011). Low-protein diet fed to pregnant mice decreases β cell mass in the F1–F3 generations at least until day 21 of life (Frantz et al., 2011), and in a separate study using a similar paradigm, F3 animals were shown to develop fasting hyperglycemia in adulthood (Benyshek et al., 2006).

The mechanisms responsible for transgenerational effects in developmental programming are poorly understood in mammals. In the case of transmission via the maternal germline, several possibilities bear mentioning: transmission via alteration of the epigenome (somatic or germline), transmission through factors in the ooplasm (such as mitochondria), or development of the F2 generation in a suboptimal uterine environment provided by the reprogrammed F1 female generation. The last point bears repeating: as both IUGR and maternal obesity have been shown to induce metabolic abnormalities in female F1 offspring, and as this altered metabolic state persists even after the F1 daughter becomes pregnant, this can in turn lead to the development of metabolic abnormalities in the F2 generation, and so forth, thus creating a vicious cycle. In principle, such effects can be experimentally ruled out by transferring the oocytes or fertilized zygotes from F1 animals into control recipient females to separate placental effects from alterations in the F1 oocyte epigenome or mitochondria. However, oocyte and embryo transfer processes themselves have been shown to have adverse effects on the offspring, thus making interpretation of these studies difficult.

Paternal Effects

Although the impact of maternal environment on children has long been clear, the father's contribution to phenotypes in the offspring is much murkier. Although paternal preconception diet has been the subject of some study, most such studies typically focus on fertility-related measures such as sperm count and motility, as it is generally believed that sperm contribute nothing but a single haploid genome complement to offspring. However, this view is changing as a burgeoning number of studies have linked paternal environmental conditions—largely stress and dietary paradigms—to offspring phenotypes. Indeed, in humans, paternal body mass appears to be a better predictor of childhood metabolic traits than maternal BMI (Figueroa-Colon et al., 2000). Paternal effect paradigms are of great mechanistic interest, as the basis by which paternal environment influences offspring is unknown at present. Although stress and toxin-related paradigms both have been documented to induce paternal effects on offspring phenotype in mammals (Rando, 2012), here we will limit our focus to dietary paradigms.

Male Line Effects in Human Epidemiological Studies

As with maternal effects, paternal effects have been described in multiple mammalian species. In humans, the best-known evidence for male line effects of nutrient availability on future generations comes from the Overkalix cohort (Bygren et al., 2001; Kaati et al., 2002; Pembrey et al., 2006). In this remote Swedish town, historical crop records were used to infer nutritional access among the ancestors of the current population. Food supply in grandparents was linked to mortality rates two generations later in a gender-specific manner—paternal grandfathers' food supply was associated with altered metabolism in grandsons, whereas paternal grandmothers' food supply was linked to outcomes in their granddaughters. Interestingly, in both cases, relative protection from, or increased risk of, metabolic disease was dependent on the age of grandparental exposure to inadequate diet. Specifically, inadequate diet in early adolescence (ages 9–13) was correlated with decreased risk of mortality in grandchildren, whereas the same exposure experienced by young adults (ages 18–22) was associated with increased risk. This finding is of significant interest, as some rodent studies also find contrasting metabolic outcomes depending on the animal's age at the time of administration of an altered nutritional environment (see below).

Although the number of human studies focused on male-line effects is relatively scant, and the numbers of individuals studied tend to be small, the concordance between such male-line epidemiological results and paternal effect studies in rodent models suggests that links between paternal diet and offspring metabolism are potentially evolutionarily conserved.

Rodent and Other Model Systems

A large and increasing number of rodent models also find effects of paternal diet on offspring metabolism. The dietary paradigms used range from relatively poor diets, such as caloric restriction and low-protein diet, to diets of nutrient excess such as high-fat or "Western" diets. The time of exposure to diet varies considerably between studies—while a subset of paternal effect paradigms focus on the period from weaning to sexual maturity, a larger number of studies focus on in utero undernutrition, in which the males used as the paternal generation were carried by mothers subject to starvation during pregnancy. These of course are a subset of the "F2" effects described above for maternal effect paradigms (Figure 1), in which the sons in such experiments are then considered the F0 generation for a paternal effect study. The timing of exposure is a key factor to take into account when considering paternal effect paradigms, as primordial germ cell (PGC) development occurs during the last week of gestation in male mice. A number of major epigenomic

transitions, such as erasure of previous imprints and establishment of male-specific cytosine methylation patterns, occur during this period (Feng et al., 2010), meaning that dietary paradigms starting after birth (generally after weaning) are presumably less likely to influence the epigenome. That said, much remains to be learned about the plasticity of the epigenome and the ability of spermatogonial stem cells to respond to environmental alterations.

Many different metabolic phenotypes have been reported to change in offspring in response to paternal diet (see below for references). The most common metabolic phenotypes measured are related to glucose homeostasis and include fasting glucose, glucose clearance, insulin release in response to glucose, and glucose clearance in response to insulin. Beyond glucose metabolism phenotypes, cholesterol and lipid metabolism, and other cardiovascular phenotypes such as blood pressure, are reportedly altered in response to paternal dietary conditions.

We will focus first on paternal diets that impact glucose metabolism phenotypes in offspring, as these are easily the most commonly reported effects of paternal diets. Perhaps the best-studied cases of paternal nutritional effects on glucose homeostasis come from in utero undernutrition paradigms. Here, pregnant females are subject to severe caloric restriction (in some paradigms, up to 50% reduction in caloric intake) during the last week of gestation, and their sons are then maintained on control diet and mated to control females. It is worth noting that this paradigm differs substantially from many of the maternal effect paradigms described above, as the starvation used is unusually severe and is only used during the last week of pregnancy. Male offspring born following this intervention—effectively, an IUGR "F2" generation transmitted via the paternal germline (Figure 1)—exhibit impaired glucose tolerance, with a 22% increase in the area under the curve following intraperitoneal glucose challenge (Jimenez-Chillaron et al., 2009). These offspring also secrete ~40% less insulin in response to glucose challenge than control offspring at 4 months of age. Other phenotypes, such as insulin resistance, were observed only in matings between both a male and a female subject to in utero undernutrition, but not in matings with either a control father or mother.

Paternal high-fat diets also induce metabolic phenotypes in offspring. Male rats maintained on a high-fat diet throughout adulthood are reported to sire daughters with mildly impaired glucose tolerance and abnormal pancreatic morphology (Ng et al., 2010). In mice, fathers consuming a high-fat diet (with or without low doses of streptozotocin in two distinct studies) sired both sons and daughters exhibiting impaired glucose tolerance (Fullston et al., 2013; Wei et al., 2014). Finally, paternal consumption of low-protein diets (9% versus 18% protein) has been linked to impaired glucose tolerance in both male and female offspring (Watkins and Sinclair, 2014).

Beyond glucose-related phenotypes, several other metabolic changes have been reported in paternal effect paradigms. Most notably, several studies have shown a link between lipid and cholesterol metabolism and paternal diet. For example, 3-week-old offspring of males consuming a low-protein diet exhibit significantly decreased hepatic levels of free cholesterol and cholesterol esters relative to control offspring, along with increased hepatic expression of genes encoding the cholesterol biosynthesis pathway (Carone et al., 2010). Cholesterol and lipid biosynthesis genes also change in expression in embryonic day E16.5 offspring of males subject to in utero undernutrition, although apparently, in this system, offspring show the opposite phenotype, with increased hepatic cholesterol stores and decreased expression of many components of the cholesterol biosynthesis pathway (Radford et al., 2012, 2014). It will be interesting to determine whether this difference stems from the timing of exposure to poor diet—in utero versus post-weaning—or reflects the different ages at which offspring were analyzed. Finally, low-protein feeding has also been shown to influence systolic blood pressure in offspring (Watkins and Sinclair, 2014). In general, many paternal effect studies will need to be repeated in additional cohorts and in different strains of mice and rats to assure reproducibility and generalization of these findings.

No doubt deeper metabolic phenotyping will continue to uncover additional effects of paternal dietary conditions on offspring metabolism. That said, perhaps the most curious aspect of metabolic effects observed following paternal dietary paradigms is that, by and large, the phenotypes observed in these studies overlap substantially with outcomes of maternal dietary intervention (see above), raising the question of whether paternal dietary interventions may alter fetal provisioning by the mother (discussed below).

Paternal Effect Models and the Sperm Epigenome

The male contribution to offspring often amounts to little more than the haploid genome complement in sperm, and as a result, a great deal of attention in paternal effect paradigms has focused on so-called epigenetic information carriers in sperm. That said, it is worth noting that fathers can contribute additional information to offspring—such information carriers minimally include (1) paternal transfer of microbiota to mates or to offspring, (2) effects of seminal fluid on maternal behavior or physiology, and (3) cryptic maternal effects in which females judge males and adjust their resource allocation to offspring accordingly (Curley et al., 2011; Pryke and Griffith, 2009). We will first review dietary effects on the sperm epigenome and then will discuss alternative information carriers below.

Five major classes of epigenetic information carriers—transcription factor abundance, chromatin state, small RNAs, DNA modifications such as cytosine methylation, and prions—have been defined in paradigms ranging from microbial environmental memory to metazoan cell state inheritance to transgenerational inheritance systems in plants and worms (Rando, 2012; Rando and Verstrepen, 2007). Of these, the most commonly studied in paternal dietary paradigms in mammals are cytosine methylation and small RNAs, which will be reviewed here.

Cytosine Methylation. Cytosine methylation, which plays well-established roles in epigenetic inheritance paradigms in plant and mammal models, has been the best-studied epigenetic mark in various paternal effect experiments. Cytosine methylation changes are often reported in offspring of paternal effect paradigms, often at loci plausibly linked to offspring physiology—in our system, offspring of low-protein fathers exhibit ~10% cytosine methylation changes across an enhancer of the key lipid regulator *Pparα* (Carone et al., 2010). However, in this case and many others, methylation changes observed in offspring

tissues are often absent in analyses of sperm samples, ruling out the simplistic hypothesis in which diet influences the sperm epigenome, which then escapes erasure during early development. For this reason, we focus here on analyses of cytosine methylation patterns in treated versus control sperm and ignore isolated reports of cytosine methylation in offspring tissues. Sperm cytosine methylation patterns have been reported to change at a number of loci in both an in utero undernutrition paradigm (Radford et al., 2014) and in response to paternal prediabetes (Wei et al., 2014). In the former case, 166 differentially methylated regions (DMRs) were identified using MeDIP-seq in pooled sperm samples, and bisulfite validation (n of ~12 animals per treatment) of 32 DMRs confirmed 17 regions of hypomethylation in the undernutrition cohort. In general, these regions exhibited ~20% changes in methylation over ~5 adjacent CpGs, with most cases apparently changing from ~40% methylation at each CpG to ~20% methylation. In the case of paternal prediabetes (Wei et al., 2014), thousands of DMRs were reportedly identified by MeDIP-seq, although validation by bisulfite conversion was only carried out for a small number of loci in pairs of animals. As in the case of in utero undernutrition, methylation differences were generally modest—changes of ~20% between sperm samples. In a handful of these cases, the cytosine methylation changes observed in sperm are also found in F1 tissues of interest, which is potentially consistent with the hypothesis that these methylation changes are inherited at fertilization and subsequently play causal roles in establishing the reprogrammed state.

However, several considerations dampen enthusiasm for the hypothesis that the reported cytosine methylation changes play causal roles in paternal effects on offspring phenotype. The primary concern is what can be called the "digital sperm problem." 10%–20% changes in cytosine methylation can be meaningful in a multicellular tissue, as for example, changing from 90% to 70% methylation at a given cytosine in a liver population could result in tripling the number of cells expressing some systemically acting growth factor. In contrast, at fertilization, each sperm truly is alone. Thus, a quantitative change in methylation should only alter the penetrance of a given phenotype: a change from 40% to 20% methylation at a specific CpG in sperm (Radford et al., 2014; Wei et al., 2014) means that one out of five, rather than two out of five, sperm carry a methylated cytosine at that position. This means that, even if the methylation status of the cytosine in question were completely responsible for some phenotype in offspring, at best, this 20% methylation change would alter the fraction of a rodent's litter expressing the phenotype from two out of five to one out of five. Thus, a 20% change in methylation at a cytosine is unlikely to result in penetrant changes in offspring. That said, given that multiple adjacent CpGs are often reported to change methylation in sperm, if such methylation changes were independent at each position, then digital sperm could plausibly carry more continuous, analog, information. Alternatively, it is also plausible that methylation changes occur specifically in a small subset of sperm competent for fertilization (with incompetent or dead sperm nonetheless polluting the ensemble methylation data) or that sperm bearing the methyl marks at this locus exhibit some competitive advantage/disadvantage (superior swimming, oocyte adhesiveness, etc.) in successful fertilization of an oocyte. Both of these scenarios would make the effective changes in methylation in fertilizing sperm much greater than those measured in the unselected sperm ensemble.

The other key challenge for cytosine methylation as a potential carrier of paternal environmental information comes from the near-global resetting of the paternal epigenome that occurs upon fertilization (Feng et al., 2010). Of course, a small subset of cytosine methylation on the paternal genome escapes erasure, with paternally contributed methylation at a subset of imprinted loci providing the canonical examples (Bartolomei and Ferguson-Smith, 2011). In addition to imprinted loci, genome-wide analyses of cytosine methylation in gametes and early embryos suggest that additional genomic regions may potentially escape this near-global reprogramming. In mice, sperm-contributed DMRs occur primarily in intergenic CpG-poor regions enriched for LINE elements and can persist for several cleavages (Smith et al., 2012). Similar enrichment of sperm-specific methylation in intergenic regions is reported in humans, although in contrast to mice, much more of this methylation appears to be erased prior to the two-cell stage (Guo et al., 2014). These results are consistent with a mechanism in which retention of H3K9-methylated histones over gene deserts in sperm (Carone et al., 2014; Samans et al., 2014) serves to recruit the maternal factor Stella to protect these regions from Tet3-mediated cytosine demethylation (Nakamura et al., 2012). Whatever the mechanism for retention of a subset of the paternal methylome, it appears to occur preferentially at distal intergenic regions rather than at, say, promoters, and in the majority of cases, methylation is lost within a few cleavage divisions. Such considerations may help guide more fruitful searches for epigenomic changes responsible for paternal dietary effects.

Beyond correlative evidence for cytosine methylation changes in paternal information transfer, functional studies must be the next step in testing the hypothesis that diet-directed methylation is responsible for offspring reprogramming. Such studies are challenging to consider, although advances in locus-specific recruitment of epigenomic regulators may allow such tests in the near future.

Small RNAs. Since the discovery of microRNAs in 1993 (Lee et al., 1993), an ever-expanding universe of small (<40 nt) RNAs have been described and shown to function in a large variety of biological paradigms. Most interestingly, small RNAs have been implicated in several well-established epigenetic inheritance paradigms, including RNAi in nematodes (Fire et al., 1998), paramutation in maize (Arteaga-Vazquez and Chandler, 2010), and many related epigenetic silencing paradigms in models from *Arabidopsis* (Chan et al., 2004; Zilberman et al., 2003) to fission yeast (Grewal, 2010). Small RNA families include well-studied species such as microRNAs, siRNAs, and piRNAs, as well as less-characterized entities, including tRNA fragments (tRFs) and enhancer-derived RNAs (eRNAs) (Ghildiyal and Zamore, 2009; Sobala and Hutvagner, 2011). In mammals, microinjection of small RNAs into zygotes has been reported to alter coat color phenotypes (Rassoulzadegan et al., 2006), cardiac hypertrophy (Wagner et al., 2008), and other phenotypes.

Several paternal effect studies in mammals have documented changes in small RNA profiles in the sperm of treated versus control males. The majority of such studies focus on microRNAs,

either because they use microRNA-specific profiling methods (such as microarrays or <24 nt size selection prior to deep sequencing) or because the more extensive literature on microRNAs makes predictions of functional consequences of these changes easier than predictions of the function of a given piRNA or tRF in zygotes. Changes in levels of specific microRNAs have been reported in sperm in response to paternal conditions both in stress-related and in some nutritional paradigms (Rodgers et al., 2013). Moreover, in the case of early paternal stress, microinjection of a total RNA pool from the sperm of stressed animals was reported to induce a subset of the phenotypes observed in offspring generated by natural mating (Gapp et al., 2014). This result suggests that some component of the RNA payload of mature sperm is capable of altering offspring phenotype when introduced into the early embryo.

That said, the hypothesis that sperm RNAs could be responsible for programming offspring phenotype presents several challenges for current mechanistic models for small RNA function. First, mammalian sperm carry extremely low levels of RNA and, considering the volume of a sperm relative to the oocyte, suggests that sperm are unlikely to carry enough RNAs to significantly alter the concentrations of a given RNA species in the oocyte, unless the RNA in question is absent or nearly so from the oocyte. This concern could be alleviated were sperm RNAs to be uniquely modified or pre-bound by an effector protein, but in any case, the simplest model of sperm delivering a pool of soluble small RNAs must contend with the issue of the miniscule sperm cytoplasm. Second, even for uniquely sperm-delivered RNAs, the biochemistry of mammalian Ago proteins strongly supports the idea that only the ~20 or so most abundant microRNAs in a given cell are likely to have regulatory impact on the cell (Wee et al., 2012), an idea that is supported by functional studies in somatic cells (Mullokandov et al., 2012) and in zygotes (Amanai et al., 2006). Finally, in mammals, it is unclear how small RNAs introduced at fertilization would have lasting effects on offspring phenotype. In the species in which transgenerational inheritance based on small RNAs is best characterized—worms, plants, and fission yeast—RNA-dependent RNA polymerase plays a key role in amplifying small RNA signals. In contrast, mammals do not encode a known RNA-dependent RNA polymerase, suggesting that whatever effects sperm RNAs have on offspring must likely occur within the first few cleavage divisions and induce longer-lasting effects by their actions during this stage.

Thus, although small RNAs represent a very strong candidate for the molecular basis of dietary information in sperm based on their roles in other model organisms, many mechanistic questions remain to be resolved to consider sperm delivery of small RNAs to be a credible influence over offspring metabolism.

Synthesizing Maternal and Paternal Dietary Paradigms. Perhaps one of the most striking aspects of the literature on ancestral dietary conditions is the extensive overlap in phenotypes induced by maternal and paternal effect paradigms. As detailed above, both maternal and paternal effect paradigms influence glucose homeostasis, cholesterol metabolism, and cardiovascular parameters in offspring. In stress paradigms, anxiety-related behaviors are often altered in offspring, again both via maternal and paternal transmission. Although these overlapping outcomes of parental exposure history could potentially reflect investigator biases—if you alter the diet of a parent, it makes sense to look for metabolic outcomes in offspring—in many of the studies detailed above, genome-wide profiling methods were applied to offspring, with metabolic phenotypes being identified in a relatively unbiased manner. Thus, although these overlaps may not be meaningful, it is nonetheless worth considering the hypothesis that maternal and paternal dietary paradigms operate via a shared downstream mechanism.

Several mechanisms could unite paternal and maternal effects on offspring. For example, numerous recent studies implicate the gut microbiota in influencing metabolism, so in principle, both males and females subject to overnutrition (for instance) could potentially inoculate their offspring with similar microbial communities that efficiently extract nutrients from food. However, in many paternal effect experiments, males are removed from the female's cage after only 1 or 2 days of mating; moreover, in our system, we have found that paternal dietary information can be transmitted to offspring even when using in vitro fertilization (J. Shea and O.J.R., unpublished data), thus removing any interaction between the male and his offspring. This last experiment also argues against a mechanism in which females judge the quality of their mate and alter resource provision to the offspring (Pryke and Griffith, 2009) and makes seminal fluid—which can significantly impact offspring metabolism in mammals (Bromfield et al., 2014)—similarly unlikely.

How then might sperm alter maternal resource provisioning? Intriguingly, numerous studies have found effects of brief embryo culture on metabolic phenotypes in offspring (Rinaudo and Wang, 2012). The earliest cell-fate decision in mammalian embryos is cell-fate allocation between the inner cell mass (ICM) and the trophectoderm (TE) of the blastocyst, which give rise to the embryo and to the extraembryonic tissues, respectively. It is thus theoretically possible that molecular changes in sperm induced by paternal diet somehow influence cell fate allocation between ICM and TE—by altering cell-cycle dynamics for the first few cleavage divisions, for instance—or alternatively, TE cell function, with resulting effects on placental development then resulting in metabolic changes in offspring as detailed above. Indeed, paternal diet and exercise have been linked to preimplantation growth dynamics (McPherson et al., 2013), potentially providing a link between paternal diet and the findings of common maternal effect phenotypes in offspring. Such a link would also help explain how epigenetic marks in sperm such as cytosine methylation or small RNAs, which generally are erased or at least not copied in the embryo, might exert long-term metabolic effects despite operating during a limited time window.

Conclusions

The combined epidemiological, clinical, and animal studies clearly demonstrate that the intrauterine environment influences both growth and development of the fetus and the subsequent development of adult diseases. There are specific critical windows during development, often coincident with periods of rapid cell division, during which a stimulus or insult may have long-lasting consequences on tissue or organ function postnatally. In maternal effect models, mitochondrial dysfunction and oxidative stress are among the earliest molecular events described in offspring subjected to nutrient restriction or

uteroplacental insufficiency and provide a strong candidate mechanism in the pathogenesis of the fetal origins of adult disease. Later in life, a number of changes in epigenetic marks have been identified in multiple tissues in offspring, which provide the likeliest mechanism by which early molecular changes result in persistent phenotypic changes. There are likely to be many additional mechanisms that will be uncovered as new tools become available to more robustly study these questions.

The near future promises advances on at least three fronts. First, the burgeoning field of epigenetic epidemiology is in its early days, but surveys of epigenetic marks in children who experience adverse placental environments promise to yield a wealth of knowledge regarding the mechanisms responsible for long-term metabolic reprogramming. A number of potential issues with extant studies exist, as for example, birth weight is only one marker of an adverse fetal environment, and confining studies to this population only may lead to erroneous conclusions regarding etiology. But this approach has great promise, particularly as it grows in scope and sophistication. Second, advances in epigenomics and in epigenetic ''editing'' in model organisms should continue to provide mechanistic insights regarding the molecular basis for transgenerational inheritance and to offer fundamental insights into early development. Finally, prevention of metabolic abnormalities will of course be one of the key goals for future efforts, as mitochondrial function and even epigenetic marks provide promising candidates for therapeutic intervention, and research efforts should be focused in this area.

ACKNOWLEDGMENTS

O.J.R. is supported by NIH grants R01HD080224 and DP1ES02545, and March of Dimes grant FY13-1268. R.A.S. is supported by NIH grants R01DK55704, RO1DK062965, and R01ES023284 and March of Dimes Prematurity Research Center.

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Cell

Leading Edge
Review

Promoting Health and Longevity through Diet: From Model Organisms to Humans

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http://dx.doi.org/10.1016/j.cell.2015.02.020

Reduced food intake, avoiding malnutrition, can ameliorate aging and aging-associated diseases in invertebrate model organisms, rodents, primates, and humans. Recent findings indicate that meal timing is crucial, with both intermittent fasting and adjusted diurnal rhythm of feeding improving health and function, in the absence of changes in overall intake. Lowered intake of particular nutrients rather than of overall calories is also key, with protein and specific amino acids playing prominent roles. Nutritional modulation of the microbiome can also be important, and there are long-term, including inter-generational, effects of diet. The metabolic, molecular, and cellular mechanisms that mediate both improvement in health during aging to diet and genetic variation in the response to diet are being identified. These new findings are opening the way to specific dietary and pharmacological interventions to recapture the full potential benefits of dietary restriction, which humans can find difficult to maintain voluntarily.

Introduction

The discovery that aging can be ameliorated by dietary, genetic, and pharmacological interventions has opened up the prospect of a broad-spectrum, preventive medicine for aging-related diseases (Table 1) (Fontana et al., 2014; Goldman et al., 2013; Partridge, 2010). Single-gene mutations that extend animal lifespan can ameliorate natural, age-dependent loss of function (Metaxakis et al., 2014; Stein and Murphy, 2012) and the pathology of aging-related diseases, including neurodegeneration (Cohen et al., 2009; Killick et al., 2009; Menzies and Rubinsztein, 2010; Pinkston-Gosse and Kenyon, 2007; Stöhr et al., 2013). Furthermore, laboratory animal models of slowed aging, naturally long-lived species such as the naked mole rat, and some humans that achieve the age of 100 have all demonstrated that a long life is not inevitably associated with late-life disability and disease (Ikeno et al., 2006; Edrey et al., 2011; Ailshire et al., 2014). Recent work has shown that specific dietary interventions can also promote long life and healthy old age.

Dietary restriction (DR), implemented as chronic and coordinate reduced intake of all dietary constituents except vitamins and minerals, was first shown 80 years ago to extend lifespan in rats. DR in both rats and mice improves most aspects of health during aging (Fontana et al., 2010a; Ikeno et al., 2006; Maeda et al., 1985). Exceptions include resistance to infection and wound healing. However, these conditions rapidly improve with re-feeding, and DR animals can then outperform controls (Kristan, 2008; Hunt et al., 2012). DR can produce substantial benefits with, for instance, ~30% of DR animals dying at old ages without gross pathological lesions, compared with only 6% of ad-libitum-fed controls (Ikeno et al., 2006). DR started in young, adult Rhesus monkeys greatly improves metabolic health; prevents obesity; delays the onset of sarcopenia, presbycusis, and brain atrophy; and reduces the risk of developing and dying of type 2 diabetes, cancer, and cardiovascular disease (Colman et al., 2014; Mattison et al., 2012).

In humans, severe food restriction without malnutrition results in many of the same physiological, metabolic, and molecular changes associated with DR in animals, including less age-associated myocardial stiffness and autonomic dysfunction, lower core body temperature, and downregulation of the *pi3k/akt/foxo* and inflammatory pathways in skeletal muscle (Cava and Fontana, 2013; Mercken et al., 2013). Furthermore, humans voluntarily undertaking long-term DR score lower than controls on multiple risk factors for cardiovascular disease and cancer (Fontana et al., 2010b). In short-term, randomized clinical trials in aging humans, DR improves several markers of health (Heilbronn et al., 2006; Fontana et al., 2010b). However, severe DR with adequate nutrition (i.e., consuming at least 100% of the RDI for each essential nutrient) is not an option for most people because it is difficult to practice and sustain and, with inadequate nutrition, can increase the risk of impaired menstrual and reproductive function, osteoporotic bone fractures, anemia, and cardiac arrhythmias (Fairburn and Harrison, 2003). Dietary interventions that avoid unrealistic levels of self-deprivation, and pharmacological interventions that recapture beneficial effects of DR, are therefore important goals to improve human health during aging.

Table 1. Interventions Extending Mean and/or Maximal Lifespan in Wild-Type Mice Fed Normal Chow

	Max Lifespan	Mean Lifespan	Main Mechanism of Action
Dietary Interventions			
Calorie restriction	yes	yes	↓ nutrient-sensing pathways
Intermittent fasting	yes	yes	↓ nutrient-sensing pathways
Protein restriction	no	yes	↓ nutrient-sensing pathways
Methionine restriction	yes	yes	↓ nutrient-sensing pathways
Tryptophan restriction	yes	yes	↓ nutrient -sensing pathways
Physical Exercise Interventions			
Endurance exercise	no	yes	↑ insulin sensitivity; ↑ AMPK ?
Genetic Interventions			
Ames and Snell dwarf	yes	yes	↓ nutrient-sensing pathways
GH receptor KO	yes	yes	↓ nutrient-sensing pathways
IGF-1 R KO	yes (in F)	yes (in F)	↓ nutrient-sensing pathways
Klotho TG	yes (in M)	yes	↓ nutrient-sensing pathways
Fat Insulin Receptor KO	yes	yes	↓ nutrient-sensing pathways
Insulin Receptor Substrate 1 KO	yes (only F)	yes (only F)	↓ nutrient-sensing pathways
Brain IRS-2 KO	yes	yes	↓ nutrient-sensing pathways
PAPP-A KO	yes	yes	↓ nutrient-sensing pathways
Ribosomal S6 protein kinase-1 KO	yes (only F)	yes (only F)	↓ nutrient-sensing pathways
FGF-21TG	yes	yes	↓ nutrient-sensing pathways
miR-17TG	?	yes	↓ nutrient-sensing pathways
DGAT1KO	yes (only F)	yes (only F)	↓ nutrient-sensing pathways
p66shc KO	yes	yes	↓ growth factor-mediated signaling
ATG5 TG	yes	yes	↑ autophagy
Type 5 Adenylyl Cyclase KO	yes	yes	↓ β-adrenergic signaling
Angiotensin II type 1 receptor KO	yes	yes	↓ angiotensin receptor signaling
Catalase targeted to mitochondria TG	yes	yes	↓ oxidative stress (mitochondrial)
Ink4/Arf-TG/TG	no	yes	↓ mitogenic over-stimulation and cell proliferation
C/EBP β/β	yes	yes	↑ mitochondrial biogenesis in white fat cells
Mclk1KO	yes	yes	↓ age-dependent loss of mitochondrial function
Hcrt-UCP2 TG	no	yes	?; ↓ core body temperature
Macrophage migration inhibitory factor KO	yes	yes	↓ inflammation; ↓ insulin pathway
E-DNIκB TG	?	yes	↓ inflammation; ↓ insulin pathway
PKA RIIβ KO	yes	yes	↓ IGF signaling?
RasGRF1 KO	yes	yes	↓ nutrient-sensing pathways
Sirt6 TG	yes (only M)	yes (only M)	↓ nutrient-sensing pathways (IGF)
Brain-specific Sirt1 TG	yes (only F)	yes	↑ mitochondrial function via Sirt1/Nkx2-1/Ox2r
TRPV1 pain receptor KO	yes (only F)	yes	modulation of CRTC1/CREB activity
Pharmacological Interventions			
Rapamycin	yes	yes	↓ nutrient-sensing pathways (mTOR)
Acarbose	yes	yes	↓ IGF signaling and ↑ FGF-21
Metformin	no	yes	↓ nutrient-sensing pathways (AMPK)
Aspirin	no	yes (only M)	↓ inflammation
Nordihydroguaiaretic acid	no	yes (only M)	↓ inflammation and oxidative stress
Green tea extract	no	yes (only F)	↓ oxidative stress
17α-Estradiol (non-feminizing estrogen)	no	yes (only M)	? non-genomic actions
Methylene Blue	no	yes (only F)	↑ mitochondrial function
Metoprolol	no	yes (in M)	↓ β-adrenergic receptor signaling
Nebivolol	no	yes (in M)	↓ β-adrenergic receptor signaling

DR increases healthy lifespan in many shorter-lived organisms, including budding yeast *Saccharomyces cerevisiae*, the nematode worm *Caenorhabditis elegans*, and the fruit fly *Drosophila melanogaster* (Figure 1). The experimental tractability of yeast and invertebrates facilitates discovery of the—often evolutionarily conserved—mechanisms through which genetic and environmental intervention improve health during aging. The mechanisms mediating the health benefits of DR are not fully

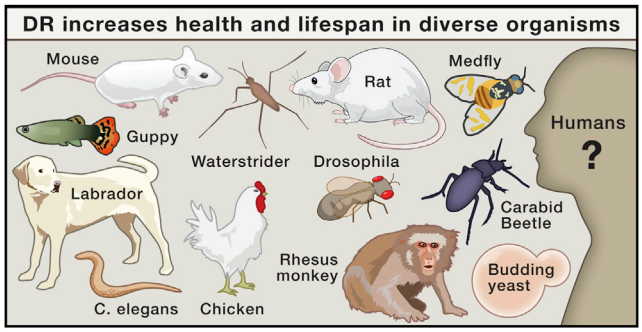

Figure 1. Dietary Restriction Increases Healthy Lifespan in Diverse Single-Celled, Invertebrate, and Vertebrate Animals

understood in any organism. A wide range of interventions has been used to impose DR, even within single species, and the mechanisms through which they extend lifespan can differ (Bass et al., 2007; Cypser et al., 2013; Mair and Dillin, 2008; Tatar et al., 2014). Multiple neural, systemic, tissue-specific, and cell-autonomous mechanisms are involved (Figure 2). In both *C. elegans* and *Drosophila*, altered sensory perception alone may play a role, with specific subsets of sensory, including olfactory and gustatory, neurons influencing lifespan (Alcedo and Kenyon, 2004; Allen et al., 2014; Apfeld and Kenyon, 1999; Ostojic et al., 2014; Waterson et al., 2014). In *C. elegans*, *Drosophila*, and mice, neural circuits both detect nutrient status and control the responses to it, in mice mediated mainly by the hypothalamus (Dacks et al., 2013). Alterations in the following also play important systemic roles: the levels of metabolites (Chin et al., 2014), the activity of the nutrient-sensing insulin/igf signaling network (Johnson et al., 2013; Kenyon, 2010) in *C. elegans*, steroid hormone signaling (Thondamal et al., 2014), and growth hormone in the mouse (Johnson et al., 2013; Kenyon, 2010). Cellular effector processes can include enhanced genomic stability and chromatin remodeling (Dang et al., 2014); improved chaperone-mediated protein homeostasis and cellular turnover processes, including autophagy (Singh and Cuervo, 2011); and increases in various forms of stress resistance (Hine et al., 2015). Molecular effectors that have been shown to mediate the effects of DR on health and longevity include FOXO (Tullet et al., 2008; Webb and Brunet, 2014), TOR (Kapahi et al., 2010; Johnson et al., 2013), AMPK (Greer et al., 2007; Burkewitz et al., 2014), sirtuins (Mouchiroud et al., 2013; Guarente, 2013), HSF, and NRF2 (Akerfelt et al., 2010; Martín-Montalvo et al., 2011). Inhibition of AKT activates FOXO, a transcription factor that upregulates several "longevity pathways" controlling DNA repair, autophagy, antioxidant activity, stress resistance, and cell proliferation (Webb and Brunet, 2014; Wang et al., 2014). Inhibition of mTORC1 improves proteostasis, increases autophagy, and enhances stem cell function (Kapahi et al., 2010; Efeyan et al., 2012; Johnson et al., 2013). Systemic or tissue-specific overexpression of some sirtuins (i.e., SIRT1, SIRT3, and SIRT6) also increases genomic stability, reduces NF-kB signaling, and improves metabolic homeostasis through histone deacetylation (Mouchiroud et al., 2013; Guarente, 2013). Moreover, activation of SIRT1 and AMPK activates PGC-1a, a transcriptional regulator of mitochondrial function, antioxidant defenses, and fatty acid oxidation (Wu et al., 1999). Activation of transcription factors heat shock factor 1 and Nrf2, by upregulating HSP70, p62, and the transcription factor ATF3, induces several antioxidant and drug-metabolizing enzymes, prevents the age-dependent impairment of proteostasis, and promotes the maintenance of cell structure, redox, and intermediary metabolism (Akerfelt et al., 2010; Martín-Montalvo et al., 2011). Multiple, parallel processes thus contribute to the increase in health during aging from DR, and the relative contribution of these may vary between DR regimes and organisms.

Interestingly, recent work has revealed the importance of timing of food intake, the role of specific nutrients, the nature of the effector mechanisms, the longer-term—including transgenerational—consequences of diet, and the key role played by the gut microbiota (Figure 3). These new findings have pointed to less drastic dietary manipulations that could be combined with pharmacological interventions to improve health and prevent disease during aging.

Meal Frequency and Timing

Only recently have humans and domesticated animals had constant access to food. During their evolution, many animals and humans ate only intermittently. For many microorganisms and invertebrates, long periods of starvation are normal and, correspondingly, many of them (including *C. elegans*) have evolved forms of quiescence in response to the onset of food shortage. Many of the genes that control quiescence are also important in the control of lifespan (Baugh, 2013). Interestingly, intermittent fasting (IF), with alternation of 2 days of ad libitum feeding with 2 day fasting, also extends worm lifespan, through a mechanism involving the small GTP-ase RHEB-1 and insulin/Igf signaling (Honjoh et al., 2009). Even chronic starvation extends lifespan in *C. elegans* (Kaeberlein et al., 2006; Lee et al., 2006), through mechanisms that overlap with those mediating the response to IF and that include the combined activity of FOXO, FOXA, and AP-1 transcription factors in two parallel starvation-responsive pathways (Uno et al., 2013).

In rodents, both fasting for 24 hr every other day or twice weekly extends lifespan up to 30%, independent of both total food intake and weight loss (Mattson et al., 2014). As for chronic DR, the magnitude of the life extension induced by IF can be influenced by the age of initiation and mouse genotype. In A/J mice, for example, IF started at 6 months did not increase lifespan and, when started at 10 months of age, reduced mean lifespan by 15% (Goodrick et al., 1990), although it is not clear whether a more or less severe form of IF could extend lifespan in these mice. IF can also protect against obesity, cardiovascular disease, hypertension, diabetes, neurodegeneration, and the clinical progression of several neurodegenerative diseases (Mattson et al., 2014). In contrast, although many studies report a protective effect against cancer progression (Berrigan et al., 2002; Lee et al., 2012), others suggest detrimental cancer initiation and promotion (Tessitore and Bollito, 2006). In rodents, multiple changes mediate benefits of fasting, including increased production of the neurotrophic factors BDNF and FGF2, reduced inflammation and oxidative stress, and enhanced cellular and molecular adaptive stress responses. Treatment of cells and

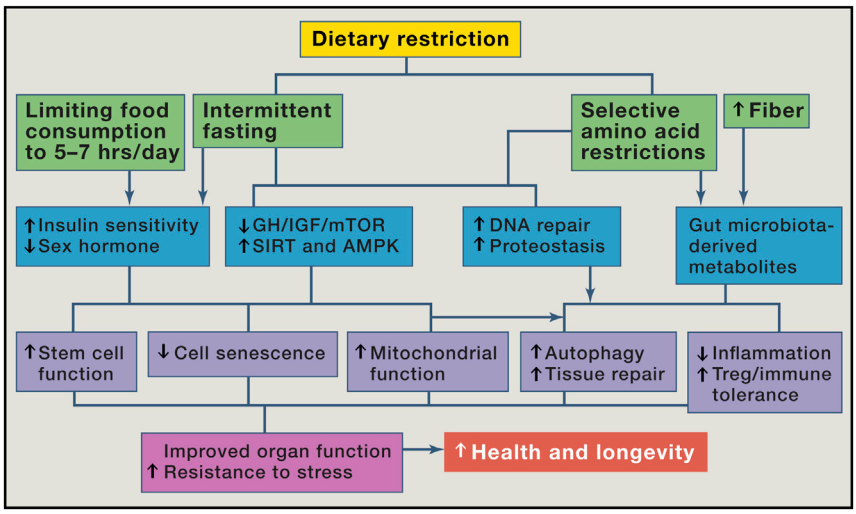

Figure 2. Dietary Restriction Modulates Multiple Systemic, Neural, and Cellular Mechanisms that Improve Health and Combat the Diseases of Aging

mice with βOHB, an endogenous histone deacetylase inhibitor powerfully induced by fasting, protects against oxidative stress (Shimazu et al., 2013). Fasting improves mitochondrial function, stimulates the production of chaperones such as HSP-70 and GRP-78, and, by inhibiting the AKT/mTOR pathway, triggers autophagy and DNA repair pathways in multiple cell types (Brown-Borg and Rakoczy, 2013; Mattson et al., 2014). Moreover, in mice, multiple cycles of fasting modulate hematopoietic stem cell protection, self-renewal, and regeneration via IGF-1 or PKA inhibition (Cheng et al., 2014). Finally, short-term fasting (1–3 days) has been shown to protect rodents against the damage induced by ischemia-reperfusion of the liver and kidney, by improving insulin sensitivity, reducing expression of markers of inflammation and insulin/igf-1 signaling, and increasing cytoprotective gene expression (Hine et al., 2015).

Many trials on the effects of IF in humans are underway. A 6 month, randomized, clinical trial in overweight or obese premenopausal women showed that fasting for 2 non-consecutive days per week results in reduced body weight, fat mass, and waist circumference and also reduced serum concentrations of total and LDL cholesterol, triglycerides, C-reactive protein, and arterial blood pressure. Several serum biomarkers of cancer risk also improved, but total and free IGF-1 did not change (Harvie et al., 2011). Similarly, in three small, short-term (8–12 weeks), randomized clinical trials in non-obese and obese individuals, alternate day fasting lowered body weight, fat mass, and risk factors for cardiovascular disease (Kroeger et al., 2014). Preliminary evidence from dogs and humans suggests that short-term fasting (24–48 hr) prior to chemotherapy reduces some chemotherapy-associated side effects by protecting normal cells, but not cancer cells, from toxicity (Safdie et al., 2009; Withers et al., 2014).

Patterns of eating over the day can also have substantial effects. Limiting daily food intake of an isocaloric diet to a 5 to 7 hr time window in humans can induce health benefits compared with a standard three to five meals per day (Mattson et al., 2014). Delaying feeding until the evening in diurnally active fruit flies, which normally eat predominantly in the morning, causes an uncoupling of their metabolic cycle from the central circadian rhythm and reduces egg laying (Gill and Panda, 2011). Time-restricted feeding of mice during 8 hr of the dark phase of the daily cycle does not affect overall calorie intake of mice on a high-fat diet but restores normal circadian rhythms of activity in metabolic pathways and protects the mice against weight gain, fat accumulation, inflammation, glucose intolerance, insulin resistance, and loss of endurance and motor coordination (Chaix et al., 2014). Humans who eat and sleep ~12 hr out of phase from their habitual patterns experience increased blood pressure, worsening of glucose tolerance, a reduction of the satiety hormone leptin, and a complete inverse pattern of the cortisol rhythm (Scheer et al., 2009). Although randomized clinical studies on effects of chronic disruption of meal patterns and circadian rhythms, as in shift workers, have yet to be performed, epidemiological data suggest an increased risk of obesity, type 2 diabetes, cardiovascular disease, cancer, and neurodegenerative diseases (Wang et al., 2011). Furthermore, 30% calorie restriction by dietary dilution, in which mice ate all day to compensate for the low energy density of the diet, had no beneficial effects on lifespan (Solon-Biet et al., 2014), possibly because of the disrupted meal pattern. Long-lived, hungry DR mice and rats consume their restricted portion of food rapidly, with an extended period of fasting (22–23 hr) between meals. Chronic DR may hence improve health at least in part through IF. Similarly, in the Wisconsin Rhesus monkey DR trial, the animals fed mainly once a day—in the National Institute on Aging (NIA) trial twice daily (Table 2)—which may have contributed to the Wisconsin DR monkeys having a 1.8× lowered rate of death from any cause (Colman et al., 2014) in contrast with the absence of a difference in death rate in the NIA study (Mattison et al., 2012) (reviewed in Cava and Fontana, 2013). Interestingly, both in overweight/obese and lean women with polycystic ovary syndrome, subjects randomized to earlier meal timing (980 kcal breakfast, 640 kcal lunch, and 190 kcal dinner) lost more weight, displayed higher insulin sensitivity, lower serum testosterone concentration, and increased ovulation rate than controls eating isocaloric diets with a later meal pattern (190 kcal breakfast, 640 kcal lunch, and 980 kcal dinner) (Jakubowicz et al., 2013).

The molecular mechanisms responsible for the effects of altered meal patterns on metabolic health are not fully understood. There may be compensatory changes in energy sensing pathways, such as AMPK, AKT/mTOR, and cyclic AMP response element binding protein (CREB), which are all implicated in cellular homeostasis and rhythmic oscillations of circadian clock targets (Mattson et al., 2014). Transgenic mice

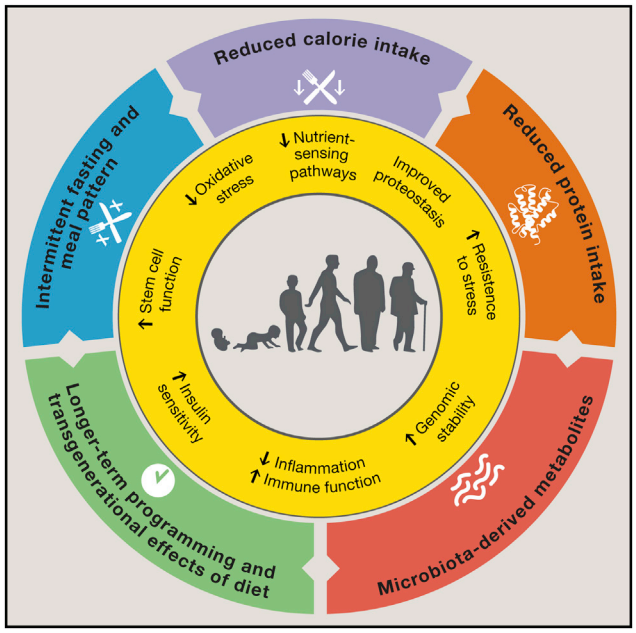

Figure 3. The Effectors of Dietary Restriction, Including Timing of Food Intake, Specific Nutrients—Especially Proteins and Particular Amino Acids—the Gut Microbiome, and Longer-Term Mechanisms, Including Developmental Programming and Trans-generational Effects, Modulate Key Mechanisms Associated with Healthy Aging

carrying a S714G mutation in hPER1, a circadian clock gene, quickly develop obesity on a high-fat diet (Liu et al., 2014). An isocaloric reduction of fat intake only during the night is sufficient to prevent obesity in these animals (Hatori et al., 2012), suggesting that altered meal pattern alone could be used to prevent certain metabolic diseases (Garaulet et al., 2013; Jakubowicz et al., 2013). Interestingly, a diet high in fat induces a rapid and reversible impairment of BMAL1 recruitment to target chromatin sites and modifies circadian transcriptome and metabolome profiles (Eckel-Mahan et al., 2013). The profound effects of timing of food intake have hence opened a promising avenue for interventions to improve humans' health during aging.

Calories or Specific Nutrients?

Determining the optimal overall intake and dietary ratios of carbohydrate, fat, and protein is challenging. The effects of reduced consumption of a specific macronutrient will depend partly upon how much of it is consumed by the controls and also upon the composition of the rest of the diet. Even for just three macronutrients, the possible combinations are vast. Combinatorial effects can be explored using a geometric framework (see the Essay by Simpson et al. (2015) on page 18 of this issue), but this is often not feasible in practice. Dietary composition can also affect overall food intake and its timing through effects on hedonistic value and satiety. Although such mechanisms are important to understand, they can also complicate analysis of the effects of nutrient intake per se. The effects of diet on health may also be age specific, a possibility that is starting to be explored experimentally in humans (Wolfe et al., 2008).

Macronutrients

Until recently, reduced intake of calories, rather than of specific macronutrients, was considered important for health benefits of DR. This assumption was primarily based on a flawed interpretation of experimental data showing that 40% calorie restriction, but not 40% protein restriction, increased lifespan in rats (Maeda et al., 1985). However, the protein-restricted rats were not food restricted, because their growth rate was normal, a point overlooked by the authors of the study. A subsequent series of studies in yeast, invertebrate model organisms and rodents has instead clearly demonstrated that a reduction in specific nutrients in the diet, rather than reduced calorie intake, is primarily responsible for improvements in health and extended lifespan, which is why we use the term DR rather than CR.

Protein and Amino Acids

Dietary guidelines from the medical literature and popular press often promulgate high protein intake, especially from animal sources rich in essential amino acids, including sulfur-containing and branched chain, to combat obesity, sarcopenia, osteoporosis, frailty, surgical stress, and mortality. However, accumulating evidence points instead to a restriction of protein or specific amino acids in the diet as promoting healthspan (Grandison et al., 2009; Solon-Biet et al., 2014; Ables et al., 2014; Nakagawa et al., 2012; Pamplona and Barja, 2006; Mirzaei et al., 2014). This conclusion initially emerged from investigation of the nutrients mediating the effects of DR and has since been amplified in broader studies of the effects of dietary composition and intake on health and lifespan.

In *Drosophila*, restriction of protein-containing yeast, but not carbohydrate or energy, extends lifespan (Mair et al., 2005). Adding back essential amino acids to the diet of DR flies decreases lifespan, with the addition of non-essential amino acids, lipid, or carbohydrate exerting little or no effect (Grandison et al., 2009). In mice, also, health and lifespan are strongly affected by the protein component of the diet, with median lifespan progressively increasing up to 30% as the dietary protein-to-carbohydrate ratio is decreased, despite a parallel increase in overall food intake and body fat and reduction in lean mass (Solon-Biet et al., 2014).

Dietary protein intake is an important regulator of the IGF-1/mTOR network (Efeyan et al., 2012). In humans, unlike rodents, chronic severe calorie restriction does not reduce serum IGF-1 concentration unless protein intake is also reduced (Fontana et al., 2008), suggesting that dietary protein or specific amino acid intake may be as or more important than calorie intake in modulating IGF-related biological processes and disease risk in men and women. In rodents, over-stimulation of the GH/IGF pathway accelerates aging and increases mortality, whereas downregulation slows aging, prevents cancer, and increases lifespan (Junnila et al., 2013). Moreover, serum IGF-1 concentration is inversely correlated with median lifespan in 31 genetically diverse, inbred mouse strains (Yuan et al., 2009) and with the risk of developing some common cancers in humans (Pollak, 2012). Interestingly, isocaloric restriction of protein and substitution of plant for animal proteins markedly inhibit prostate and breast cancer growth in human xenograft animal models of neoplasia, with reduced serum IGF-1 levels and downregulation of intratumor mTOR activity, histone methyltransferase EZH2, and the

Table 2. Characteristics of the University of Wisconsin and NIA DR Monkey Studies

	University of Wisconsin	National Institute on Aging
Rhesus monkeys (n)	76 (46 m, 30 f)	120 (60 m, 60 f)
Genetic origin of monkeys	India	China and India
Age at baseline	all adult	juvenile (20 m, 20 f); adolescent (20 m, 20 f); old (20 m, 20 f)
Housing	single caged	single caged
Randomization	1 CR: 1 control	1 CR: 1 control
Dietary regimen of CR monkeys	30% restriction from a BL intake assessed individually	30% restriction from BL intake levels based on NRC guidelines
Dietary regimen of control monkeys	fed ad libitum	controlled allotment of food each day to avoid obesity (~5%–10% CR)
Meal patterns	morning meal, plus 100 Kcal integration of food at late afternoon	twice a day
Source of nutrients	semi-purified diet rich in refined foods	natural ingredients (pesco-vegetarian diet)
Macronutrient composition of diets	15% protein (lactalbumin); 10% fat (corn oil); 65% carb (cornstarch, sucrose); 28.5% from sucrose; 5% fiber	17.3% protein (fish, soybean, wheat, corn); 5% fat (fish, soy oil, wheat, corn, alfalfa); 56.9% carb (ground wheat and corn); 3.9% from sucrose; 5%–7% crude fiber
Vitamin supplements	only for CR monkeys	40% of RDA in vitamin in both control and CR monkeys
Food intake measurement	daily quantification for each animal	1 week per year

associated histone mark H3K27me3, two epigenetic markers of prostate cancer progression (Fontana et al., 2013).

Within the protein component of the diet, specific amino acids or their ratio can be important for health and lifespan. Selective restriction of asparagine, glutamate, or methionine in the medium has been shown to extend chronological lifespan in yeast (Dilova et al., 2007; Wu et al., 2013a, 2013b). In *Drosophila* and rodents, restriction of methionine and tryptophan, respectively, extends average and maximal lifespan (Ables et al., 2014; Miller et al., 2005; Zimmerman et al., 2003). In the DR Rhesus monkey trials, the Wisconsin diet contained higher concentrations of methionine and branched-chain amino acids derived from lactalbumin than did the NIA diet (Table 2), which could explain some of the differences in effects on cancer and mortality.

Amino acid availability is sensed by multiple evolutionarily conserved molecular pathways, the most important being mTOR and GCN2. Activation of mTOR is modulated by different essential amino acids in a tissue-specific manner, with branched-chain amino acids playing a key role (Efeyan et al., 2012). In contrast, absence of any individual amino acid activates GCN2, which is required for the protective effect of short-term protein or tryptophan deprivation on surgical stress in mice (Peng et al., 2012). The protein/amino acid restriction-induced protective mechanisms against stress, damage accumulation, and aging downstream of TOR inhibition and GCN2 activation are unknown. Nonetheless, GCN2 activation stabilizes ATF4, a transcription factor essential for the Integrated Stress Response, which is elevated in several dietary, genetic, and pharmacological animal models of longevity (Li et al., 2014; Hine et al., 2015).

Reduced dietary methionine, in particular, induces specific, protective, molecular mechanisms. The sulfur-containing amino acids methionine and cysteine are metabolized through the trans-sulfuration pathway, lesions in which are associated with increased incidence of age-related pathologies in humans. DR in *Drosophila* results in increased activity of the rate-limiting enzyme in the trans-sulfuration pathway, and inhibition of the pathway blocks the response of fly lifespan to DR. Furthermore, transgene-mediated increase in activity of the pathway increases fly lifespan (Kabil et al., 2011). Interestingly, the trans-sulfuration pathway is the primary source of hydrogen sulfide in cells, and hydrogen sulfide can increase lifespan in *C. elegans* (Miller and Roth, 2007). Furthermore, a trans-sulfuration-pathway-dependent increase in production of hydrogen sulfide is seen in yeast, worm, fruit fly, and rodent models of DR, and in mice DR-induced resistance against ischemia reperfusion injury requires this increase (Hine et al., 2015). Interestingly, in the long-lived Ames dwarf mouse, which lacks growth hormone, expression of genes in the trans-sulfuration pathway and the flux of methionine to the pathway are increased, associated with higher levels of GSH (Uthus and Brown-Borg, 2006). The lifespan of Ames dwarf mice and of mice lacking the growth hormone receptor is extended relative to controls on normal diet but does not respond to methionine restriction and then becomes similar to that of controls (Brown-Borg et al., 2014). Thus, the increased health during aging of these somatotropic mutant mice may be at least in part attributable to increased trans-sulfuration activity.

In humans, little is known on the effects of dietary modifications of protein quantity and quality in modulating molecular pathways that control aging, stress resistance, and age-associated diseases. Nonetheless, ongoing clinical trials should soon begin to reveal the metabolic and molecular adaptations induced by protein restriction and alterations of amino acid intake in relativity healthy overweight human subjects and in cancer patients.

Microbiota-Derived Factors and Healthy Aging

In humans, only ~10% of cells and less than 1% of genes are human, and the rest come from trillions of microbes in the gastrointestinal tract. Rapidly accumulating metagenomic data indicate that altered food intake, especially protein and insoluble fiber, have rapid and profound effects on gut microbiota structure, function, and secretion of factors that modulate multiple inflammatory and metabolic pathways (Muegge et al., 2011; Clemente et al., 2012; David et al., 2014; Thorburn et al., 2014). G-protein-coupled receptors expressed by enteroendocrine and immune cells may be important mediators of the effects of the microbiome (Thorburn et al., 2014). For example, oral administration to mice of 17 non-pathogenic *Clostridia* species isolated from healthy human fecal samples results in gut microbiota that provide short-chain fatty acids, bacterial antigens, and a TGF-β-rich environment, which help differentiation, expansion, and colonic homing of Treg cells and reduce disease severity in multiple models of colitis and allergic diarrhea (Atarashi et al., 2013). In contrast, diet-induced microbiota dysbiosis is associated with increased risk of developing cardiovascular disease, obesity-associated metabolic abnormalities, cancer, and autoimmune and allergic disease (Clemente et al., 2012).

In nature, *C. elegans* feeds on a variety of bacterial species that grow on rotting vegetation, which also constitute the gut microbiome of the worm. Feeding *C. elegans* with soil bacteria, *Bacillus mycoides*, and *Bacillus soli* instead of the standard laboratory *E. coli* OP50 strain, significantly extended lifespan and stress resistance, suggesting that microbial-derived factors may modulate pro-longevity pathways (Abada et al., 2009). Moreover, wild-type *C. elegans* fed respiratory-incompetent *E. coli* show increased lifespan (Saiki et al., 2008). A comparison of the effects of *E. coli* and *Comamonas aquatica* on the *C. elegans* host identified vitamin B12 as a major diffusable factor from *Comamonas* that influenced patterns of gene expression and the rate of development and fertility of the worm (Watson et al., 2014). Interestingly, effects of chemicals on the worm can also be mediated by the gut microbiome. Metformin, the drug used as the first line of defense against type 2 diabetes, extends lifespan of *C. elegans* fed on live, but not killed, *E. coli*, and it does so by disrupting the folate cycle and methionine metabolism of the *E. coli*. In consequence, the supply of bacterial methionine to the worm is reduced, inducing a type of methionine restriction, which is consistent with the action of metformin as a DR mimetic (Cabreiro et al., 2013). The relatively simple gut microbiome of *Drosophila* is also derived from its food intake, and bacterial density and composition have a substantial effect upon the fly host (reviewed in Erkosar and Leulier, 2014). Bacterial density increases during the aging of the fly and can compromise gut integrity (Guo et al., 2014). The normal complement of gut bacteria enables the flies to use low-nutrient or unbalanced diets by providing them with B vitamins, particularly riboflavin, and by promoting protein nutrition (Wong et al., 2014).

Alterations in the gut microbiome may contribute to the improvement in health from DR and time-restricted feeding. Long-term dietary restriction of mice, with either a normal or a high-fat diet, leads to alterations in composition of the gut microbiome, although any contribution of these changes to the health of the DR mice remains to be elucidated (Zhang et al., 2013). In mice on a high-fat diet, time-restricted feeding during 8 hr of the dark phase decreased representation of *Lactobacillus* species, which are associated with obesity, and increased *Ruminococcaceae* species, which protect against metabolic disease associated with obesity (Zarrinpar et al., 2014). Experimental transplantations of microbiota associated with healthy eating could be revealing of their causal role.

Health, Disease, and Longevity on Various Timescales, Including Inter-generational

The effects of nutrition, including DR, can be exerted on timescales ranging from more or less instantaneous to inter-generational. For instance, in *Drosophila*, DR acts acutely, with flies switched between DR and ad libitum feeding almost immediately adopting the mortality pattern of a control group kept permanently in the feeding regime that the switched flies join (Mair et al., 2003). At least as reflected in their mortality rate, these flies thus have no memory of their nutritional history, and their patterns of gene expression also change rapidly in response to DR (Whitaker et al., 2014). In contrast, the mortality rates of *C. elegans* subjected to one form of DR retain a permanent memory of the previous feeding regime after a dietary switch (Wu et al., 2009). The mortality rates of mice and rats subjected to switches between DR and AL feeding later in life have shown mixed responses, but a meta-analysis suggests that there is a permanent memory of diet in these animals (Simons et al., 2013). Similarly, even short episodes of DR early in adulthood in male mice can induce a glycemic memory apparent as increased glucose tolerance (Cameron et al., 2012; Selman and Hempenstall, 2012). However, in mice and humans, acute responses to DR also occur, including improved insulin sensitivity, reduced inflammation, and protection against ischemia reperfusion injury and other surgical stressors (Hine et al., 2015).

Developmental Programming

In contrast to immediate effects of diet, in mammals (including humans), nutrition in early life (including in utero) can have lasting effects on health during aging, often referred to as developmental programming. For instance, undernourished rat and mouse mothers produce offspring with low birth weight and multiple metabolic defects, including early-life adiposity, altered pancreatic function, and progressive glucose intolerance (Tarry-Adkins and Ozanne, 2014; Vickers, 2014). Maternal effects on offspring can include changes to the composition of the egg, alterations to the environment in utero, and peri-natal effects such as transmission of the microbiome and alterations to lactation, and can be manifest in the offspring as changes in gene expression and epigenetic modifications, including DNA methylation, histone modification, and expression of microRNAs, as well as evidence of increased cellular aging (Aiken and Ozanne, 2014; Colaneri et al., 2013; Radford et al., 2012). Epidemiological data from humans also show a consistent effect of developmental programming by early—including in utero—nutrition, although the evidence on the mechanisms involved is necessarily correlational rather than experimental. The thrifty phenotype hypothesis (Hales and Barker, 2001) postulates that many of the changes in organ structure and metabolism seen in humans in response to restricted nutrition—particularly of protein—in utero can be understood as the consequences of immediate

responses of the fetus to ensure survival and spare vital organs such as the brain. Viewed in this way, an under-nourished fetus makes the best of a bad job with adverse consequences for health in later life, including reduced glucose tolerance and a higher incidence of ischemic heart disease, problems that are greatly exacerbated by subsequent adequate or over-nutrition. However, a poor functional capacity for insulin secretion would not be detrimental to individuals who continued to be poorly nourished and remained thin and, therefore, insulin sensitive, and it remains possible that some fetal and post-natal responses to low nutrition are advantageous in conditions of continuing poor nutrition.

Inter-generational Effects of Diet

Current nutrition may act as a predictor of future nutritional conditions if food availability shows local variation or if timing of natural cycles of food scarcity and abundance occurs on an appropriate timescale. Under these circumstances, information gained early in life or even in earlier generations could be profitably used to anticipate future nutritional prospects and adjust physiology accordingly (Rando, 2012). Such considerations may partly explain why inter-generational effects of diet can also be transmitted through males. The evidence for a role of epigenetic inheritance in these cases is largely correlational, and a direct experimental testing of the hypothesis is challenging (Heard and Martienssen, 2014; Rando, 2012). Cellular mechanisms by which metabolic changes can be communicated to chromatin are being increasingly discussed (Gut and Verdin, 2013; Katada et al., 2012; Lu and Thompson, 2012).

In *Drosophila*, the sugar content of the paternal diet, even over a 2 day period during which the offspring are sired, can elicit increased lipid content in offspring. Sugar in the diet de-silences chromatin-state-defined domains both in mature sperm and in offspring embryos, and H3K9/K27me3-dependent reprogramming of metabolic genes in two time windows in the germline and the zygote is required for increased lipid content of offspring. Furthermore, data from mice and humans, including discordant human monozygotic twins, show a similar signature of chromatin de-repression associated with obesity (Öst et al., 2014).

Livers of offspring of male mice fed a low-protein diet show elevated expression of genes involved in lipid and cholesterol biosynthesis. These alterations are accompanied by subtle (in the region of 20%) changes in DNA cytosine methylation in specific gene regions, including a putative enhancer for the lipid regulator Ppara (Carone et al., 2010). Mice subjected to in utero undernourishment are glucose intolerant, and they can transmit the glucose intolerance even though they themselves are not undernourished. They experience the effects of maternal undernourishment during a period that includes the time when their germ cell DNA reacquires methylation. The sperm DNA of these males is hypomethylated at multiple sites, especially ones enriched in regulatory elements and regulators of chromatin. Although these altered methylation patterns are not apparent in the tissues of their offspring, there are perturbations to gene expression, possibly attributable to other types of epigenetic alteration (Radford et al., 2014).

Evidence is also starting to point to truly inter-generational effects of diet, where information about dietary history is epigenetically transmitted in the germline in the absence of any further input from the organism or its environment. For instance, in the nematode worm *Caenorhabditis elegans*, starvation-induced developmental arrest has effects that persist for at least three generations, with the third generation offspring of the starved great-grandparents showing increased adult lifespan. The starvation event leads to the generation of small RNAs that are also inherited for at least three generations, that target the mRNAs of genes involved in nutrient reservoir activity (Rechavi et al., 2014), and that are possibly also causal in the increased lifespan of the third generation descendants. It is at present not clear whether these more persistent effects of diet represent non-adaptive perturbations to physiology, anticipatory programming of an adaptive response to nutritional circumstances, or both. Effects of nutrition of the paternal grandfather on grandchildren have been reported in humans, but the mechanisms responsible are unknown (Pembrey et al., 2014). Recent work with mice has suggested that sex-of-parent-of-origin effects may be much more pervasive and influential than previously supposed. Even though the number of imprinted genes in the mammalian genome is predicted to be small, non-imprinted genes can regulate the tissue-specific expression of many other genes differently when transmitted by females or males, possibly by physical interaction with imprinted loci (Mott et al., 2014). These findings could have profound implications for human aging and disease.

Genetic Variation in Response to Diet

Individuals of different genotypes can respond differently to diet. Although little studied outside the context of inborn errors of metabolism, such genetic effects in humans are potentially important for identifying sub-groups that would benefit from dietary modulation. Females and males often respond very differently to dietary and pharmacological interventions, and evidence is mounting for the importance of other types of genetic variation.

DR has proved to extend lifespan in most species examined, including many non-model organisms, although it has been suggested that increased lifespan in response to DR may have evolved in part as an artifact of laboratory culture (Nakagawa et al., 2012). However, strains of *C. elegans* and *Drosophila* collected directly from nature respond normally to DR (Metaxakis et al., 2014). Wild-derived mice, on the other hand, can show little or no response (Harper et al., 2006). The stresses of captivity in non-domesticated animals could be part of the explanation for this finding. In addition, wild mice may respond to milder or stronger DR than the single level used in the investigation (Gems et al., 2002). A similar experimental approach applied to recombinant inbred mouse strains, using a single, 40% reduction in overall intake showed a range of responses from a 98% extension of lifespan to a 68% reduction (Liao et al., 2010). A wider range of reductions intake would have revealed whether these strains differ only in the intake level at which their lifespan peaks under DR or whether DR indeed does not extend lifespan in some strains. In general, the lifespans of model organisms show a tent-shaped response to the level of food intake, with peak lifespan at intermediate food intake and a decline through starvation to the left and through increased levels of food intake to the right (Partridge et al., 2005). In order to determine whether

a strain or species responds to DR, a range of degrees of food intake should therefore be explored (Gems et al., 2002). The apparent lack of response of lifespan to DR trials in the NIA rhesus monkey trial may have been at least in part attributable to the 5%–10% DR applied to the control animals, which may have been sufficient to bring them close to peak lifespan.

Experimental analysis of genetic effects on the response to DR has tended to focus on major genetic variation, caused by either specific gene mutants or inbreeding (Schleit et al., 2013). Although this approach can be informative about mechanisms of the normal response to DR and hence candidates for disease prevention in humans, it may not reveal much about the effects of natural genetic variation in outbred human populations. It is becoming clear that such variation is important. For instance, interventions to reduce weight often have beneficial effects on blood lipid profiles, type 2 diabetes, and risk of cardiovascular disease, but some individuals respond poorly or not at all, thus limiting the effectiveness and increasing the cost of intervention programs. A study of the effects on blood lipid profiles and diabetes of increased exercise and lowered intake of fat revealed that a higher genetic risk score for dyslipidemia based upon SNP genotyping was associated with a substantially diminished response to intervention (Pollin et al., 2012). Studies of this kind could also throw light on underlying mechanisms and, hence, individually targeted dietary and other types of intervention that could be effective in preventing disease.

Conclusions and Future Perspectives

Recent and accumulating work in unicellular and invertebrate model organisms, rodents, monkeys, and humans indicates that diet has a much more pervasive and prominent role than previously thought in modulating mechanisms of aging and its associated diseases. More work is needed to understand the interactions among calorie intake, meal frequency and timing, single-nutrient modifications, the microbiome, and nutritional history in modulating the key mechanisms that maintain cellular, tissue, and organ function during aging. Development of biomarkers is also urgently needed to delineate the differences between an optimal dietary regime and starvation, taking into account individual variation in genotype and epigenotype. Finally, we need to discover predictive biomarkers that can be used in design of randomized clinical trials to test the efficacy of selected dietary and/or pharmacological interventions on risk of disease, including cancer, cognitive impairment, metabolic disease, frailty, and the rate of biological aging.

ACKNOWLEDGMENTS

We apologize for omission of relevant works due to space constraints. The research of L.F. is supported by grants from the Bakewell Foundation, the Longer Life Foundation (an RGA/Washington University Partnership), the National Center for Research Resources (UL1 RR024992), the Italian Federation of Sport Medicine (FMSI), and the European Union's Seventh Framework Programme MOPACT ("Mobilising the potential of active ageing in Europe"; FP7-SSH-2012-1 grant agreement number 320333). L.P. is supported by the Max Planck Society, the ERC, and the Wellcome Trust (UK). All of the authors contributed equally to conceive and elaborate upon the ideas presented in this Review. All of the authors can be contacted for discussions or clarifications.

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Cell

The Hunger Genes: Pathways to Obesity

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http://dx.doi.org/10.1016/j.cell.2015.03.008

The global rise in the prevalence of obesity and associated co-morbidities such as type 2 diabetes, cardiovascular disease, and cancer represents a major public health concern. The biological response to increased consumption of palatable foods or a reduction in energy expenditure is highly variable between individuals. A more detailed mechanistic understanding of the molecular, physiological, and behavioral pathways involved in the development of obesity in susceptible individuals is critical for identifying effective mechanism-based preventative and therapeutic interventions.

Introduction

Obesity is defined as an increase in fat mass that is sufficient to adversely affect health (Sperrin et al., 2014; Whitlock et al., 2009). While the absolute quantification of fat mass is usually only performed in the research setting, body mass index (BMI; weight in kg/height in meters^2) is a useful surrogate marker. Using the World Health Organization (WHO) definition of a BMI more than 30 kg/m^2 to define obesity, 30% of Americans and 10%–20% of Europeans are classified as obese, with the prevalence rising in many developing countries (http://www.who.int). As body mass index increases, so does the relative risk of type 2 diabetes, hypertension, and cardiovascular disease (Berrington de Gonzalez et al., 2010). Furthermore, an increase in the prevalence of childhood obesity (11%–17% in Europe and the US) has driven an increase in medical problems such as type 2 diabetes mellitus in adolescents (Fagot-Campagna, 2000). At a societal level, obesity is associated with disability, mortality, and substantial health costs. At an individual level, severe obesity is often associated with a multitude of clinical problems, including sleep disturbance and respiratory difficulties, joint and mobility issues, as well as considerable social stigma, which can affect quality of life as well as educational attainment and job opportunities (Puhl and Brownell, 2001).

In this Review, we provide a perspective on the contribution of environmental, genetic, and other factors to the development of obesity. We discuss how these factors impact the molecular and physiological mechanisms that regulate energy intake and energy expenditure in humans and highlight ongoing strategies to dissect the complex neural circuits and pathways that modulate energy homeostasis and their potential to be targeted by preventative and therapeutic interventions.

Obesity as a Disorder of Energy Homeostasis

Humans, like other mammals, are able to regulate their body weight over long periods of time despite day-to-day variation in the number of calories consumed and in levels of energy expenditure, irrespective of the level of adiposity. Fundamentally, factors that influence changes in body weight must ultimately disrupt the balance between energy intake and expenditure over time, the utilization of substrates (fat, protein, carbohydrate), and/or nutrient partitioning (storage of excess calories). Physiological studies in healthy normal weight individuals have shown that total energy expenditure decreases by an average of 10% with acute caloric restriction and increases with caloric excess (Ravussin et al., 2014). However, in humans, the homeostatic regulation of energy balance is easily overwhelmed by external stimuli. For example, in a study in which people were given free access to food, the average daily intake exceeded 150% of energy requirements. In such experimental settings, and potentially in the free-living environment, some individuals seem more readily able to resist weight change with overeating, possibly due to inter-individual variation in the energy costs of weight gain (Ravussin et al., 2014).

Environmental Factors Drive the Rise in Obesity Prevalence

The increasing prevalence of obesity worldwide (an approximate doubling in the last 30 years), the inverse relationship between obesity and socioeconomic class, and the secular trend toward increasing obesity in developing countries associated with urbanization provide clear evidence of the environmental influences on weight gain (Ogden et al., 2014; Popkin, 2006). The adoption of relatively sedentary lifestyles due to reduced physical activity at work and in leisure time coupled with an abundance of easily available, energy-rich, highly palatable foods represents a nutrition transition that, according to the World Health Organization, is now one of the greatest risk factors for ill health worldwide (http://www.hsph.harvard.edu) (Figure 1). Interestingly, some recent analyses of trends in obesity prevalence have suggested a decline or stabilization of obesity prevalence, especially in children in the US and some European countries, findings that are consistent with dynamic models using prevalence data and birth and death rates (Ogden et al., 2014; Thomas et al., 2014). However, many countries have either increasing (China) or decreasing (European countries) birth rates, so the potential global impact of these estimations is not readily predictable. Recent studies show that second-generation migrants to the US from all ethnic groups are heavier than their parents who migrated but that people from some ethnic groups are more likely to gain weight than others upon

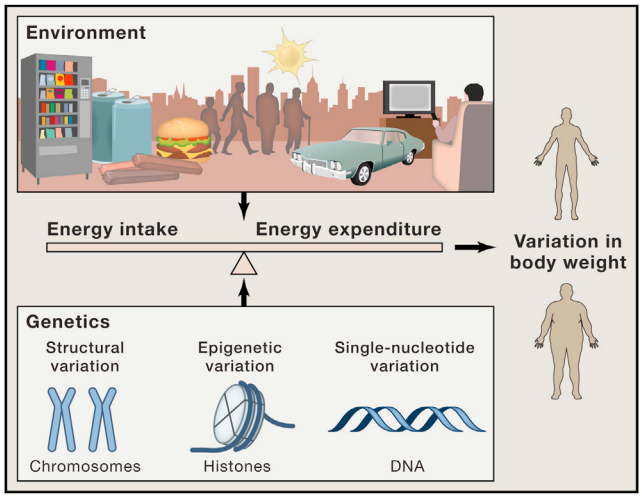

Figure 1. Contribution of Genes and Environmental Factors to Weight Gain

Human adiposity is influenced by complex interactions between genetic and environmental influences. The current environment potently facilitates the development of obesity. Abundance of highly processed food has a major impact on energy intake, whereas numerous other environmental factors, such as television watching, leisure activities, and transport, negatively affect energy expenditure. In any environment, there is a variation in body fat and BMI in large part influenced by genetic variation disrupting energy homeostasis by either decreasing energy expenditure or increasing energy intake.

transitioning to a more obesogenic environment (Singh and Lin, 2013), suggesting that, in addition to strong environmental drivers, genetic factors play a role in influencing obesity susceptibility.

Individual Susceptibility to Weight Gain Is Highly Variable—Role of Genetic Factors

In any environment, whether energy rich or energy lacking, there is considerable individual variation in body weight and fat mass, suggesting that human adiposity is influenced by complex interactions between genetic, developmental, behavioral, and environmental influences. Evidence for genetic contributions to body weight comes from family, twin, and adoption studies, which cumulatively demonstrate that the heritability (fraction of the total phenotypic variance of a quantitative trait attributable to genes in a specified environment) of BMI is between 0.71 and 0.86 (Silventoinen et al., 2008). Heritability estimates can change over time and can differ between populations. Recent studies in a UK sample of 5,092 twin pairs aged 8–11 years growing up during a time of dramatic rises in obesity confirmed substantial heritability for BMI and waist circumference (77% for both), while there was a very modest shared-environment effect, and the remaining environmental variance was unshared (Wardle et al., 2008b). Interestingly, similar heritability estimates have been found when studying monozygotic and dizygotic twins who were reared together and apart (Allison et al., 1996) and in adoption studies in which adopted children were discovered to have body sizes that were more similar to those of their biological parents than their adopted parents (Sørensen et al., 1989).

The high heritability of phenotypes related to obesity supports the contribution of genetic factors but does not indicate the number of genes or how those genes interact with environmental factors. The "thrifty gene hypothesis" suggests that we harbor genetic variants that favor efficient food collection and fat deposition to survive periods of famine and that, in the face of the easy availability of food, these genes/variants are disadvantageous. However, an alternative hypothesis is that obesity is selected against by the risk of predation. This hypothesis suggests that random mutations and genetic drift, rather than directed selection, have influenced changes in the population distribution of fat mass that may be more readily reconcilable with the findings that, even in Western societies, most people are not obese (Speakman, 2007).

Evidence for the interaction of inherited factors with changes in energy intake and expenditure was provided by landmark experimental overfeeding studies by Bouchard and colleagues, who showed that weight gain induced by overfeeding mono- and dizygous twin pairs under direct supervision was highly correlated within twin pairs but varied widely among pairs of twins (Bouchard et al., 1990). Similarly, the response to negative energy balance via an exercise regime was also heritable (Bouchard et al., 1996). Notably, the inter-twin correlations were greater for weight loss than for weight gain, suggesting tighter biological control of the response to negative energy balance.

Hypothalamic Circuits Regulating Energy Homeostasis

Ultimately, signals from cumulative genetic and environmental influences that reflect changing energy status have to be detected and integrated by brain circuits that can, through their projections, regulate energy balance. In the early 1900s, clinical reports of patients with tumors involving hypothalamo-pituitary structures associated with food-seeking behavior and obesity suggested that the hypothalamus may be involved in the regulation of body weight. Chemical and electrolytic lesioning experiments in animals in the 1930s and 1940s established the key role of the hypothalamus in the regulation of energy homeostasis. The degree of weight gain/weight loss seen in these experiments was, in part, determined by the size and precise location of the lesions, which suggested that there were specific hypothalamic circuits that promote or suppress feeding behavior (Anand and Brobeck, 1951; Hetherington and Ranson, 1940).

The hypothalamus is essentially a hub for key circuits that integrate sensory inputs; compare those inputs to basic "set points," or parameters for body temperature, electrolyte balance, sleep-wake cycle, circadian rhythms, and energy homeostasis; and then initiate a set of responses by activating autonomic, endocrine, and behavioral outputs that aim to maintain these set points (homeostasis). The hypothalamus regulates autonomic nervous system activation via neurons that directly innervate parasympathetic and sympathetic preganglionic neurons, as well as neurons in the brainstem that control autonomic reflexes. Individual pre-autonomic neurons project to multiple levels of the spinal cord, where they selectively innervate end organs such as the heart, kidney, and adipose tissue. Autonomic innervation of the pancreas contributes to the regulation of insulin and glucagon secretion.

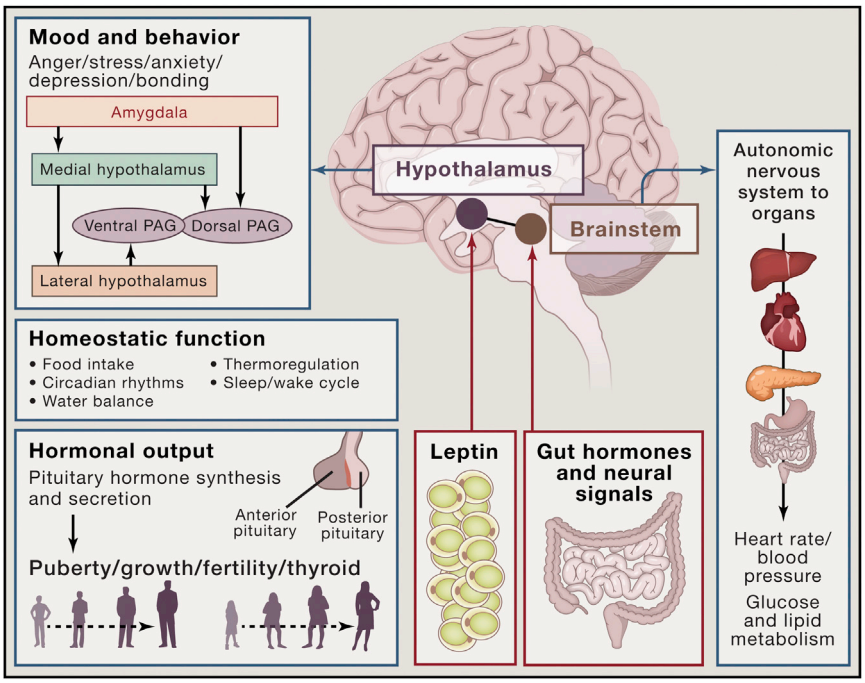

Figure 2. Leptin: A Master Regulator of Human Energy Homeostasis

The adipocyte-derived hormone leptin signals nutritional depletion and initiates a series of changes in energy intake, energy expenditure, autonomic nervous system tone, and neuroendocrine function in order to maintain energy homeostasis. The hypothalamus primarily coordinates many of these processes and also regulates circadian rhythms, temperature, and sleep. Through neuronal connections to the amygdala and periaquaductal gray (PAG) the hypothalamus also modulates a range of behaviors and moods such as stress, anger, anxiety, and aggression. Via its connections to the brainstem—direct and indirect via the cortex—neurons in the hypothalamus modulate autonomic nervous system tone which, in turn, influences many metabolic processes in peripheral tissues, such as the liver, pancreas, heart, and gut. Beyond energy homeostasis, leptin also has important effects on immune function and puberty.

Molecular Characterization of the Circuits Involved in Energy Homeostasis

While the location of the neural circuits regulating energy homeostasis was apparent from the early 1930s, a critical advance came as a result of parabiosis experiments in inbred strains of mice with severe obesity (*ob*/*ob* and *db*/*db*), which suggested the existence of a circulating factor that regulated weight (Coleman, 1973; Coleman and Hummel, 1969). The identification of this hormone, leptin, through positional cloning of the *ob* gene that was mutated in severely obese *ob*/*ob* mice (Zhang et al., 1994) paved the way for the identification and characterization of the neural circuits regulating energy homeostasis. Normalization of the phenotype of severely obese leptin-deficient *ob*/*ob* mice (characterized by increased food intake, reduced energy expenditure, hypogonadism, low thyroid hormone levels, elevated levels of corticosterone, and low blood pressure), by central leptin administration proved that leptin is a key regulator of energy homeostasis (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995).

Leptin—A Master Regulator of Human Energy Homeostasis

Early human studies showing that leptin mRNA concentrations in adipose tissue and serum leptin concentrations correlated positively and very closely with the amount of fat mass (Considine et al., 1996; Maffei et al., 1995) led to the notion that leptin's primary role was to signal increasing energy stores. However, it rapidly became clear that most people are relatively resistant to rising endogenous or exogenously administered leptin (Heymsfield et al., 1999). Instead, leptin's physiological role in humans, as in mice (Ahima et al., 1996), appears to be to signal nutritional depletion, such that fasting or weight loss results in a fall in leptin levels (Chan et al., 2003), which then triggers a series of changes in energy intake, energy expenditure, and neuroendocrine function in order to maintain energy homeostasis.

Evidence supporting leptin's role in human physiology emerged from the identification and characterization of severely obese people with homozygous loss-of-function mutations that reduce the production, secretion, or biological activity of leptin (Montague et al., 1997; Strobel et al., 1998; Wabitsch et al., 2015) or disrupt signaling through the leptin receptor (Clément et al., 1998; Farooqi et al., 2007b). While these disorders are rare, being found in 1%–5% of patients with severe obesity, their characterization has demonstrated that leptin regulates energy balance, neuroendocrine pathways, and the autonomic nervous system (Figure 2). These genetic findings have been supported and extended by elegant studies by many investigators in normal weight in the context of fasting or the weight-reduced state (Rosenbaum et al., 2002, 2005; Welt et al., 2004) and in patients with lipodystrophic syndromes characterized by relative leptin deficiency due to a loss of adipose tissue mass (Oral et al., 2002).

Impaired leptin signaling in humans is characterized by an intense drive to eat (hyperphagia), reduced sympathetic tone, mild hypothyroidism, hypogonadism, and impaired T-cell-mediated immunity, features that are reversed with the administration of recombinant human leptin in people with mutations in the leptin gene (Farooqi et al., 1999, 2002; Licinio et al., 2004; Ozata et al., 1999). Leptin also appears to be a major driver of the increase in blood pressure seen with weight gain, as blood pressure is low in obese mice and humans with disrupted leptin signaling (in contrast to diet-induced obesity in rodents/more common forms of obesity in humans) (Simonds et al., 2014).

Leptin mediates its effects by binding to the long form of the leptin receptor expressed on hypothalamic neuronal populations in the arcuate nucleus of the hypothalamus and other brain regions (Münzberg and Myers, 2005). While homozygous mutations that disrupt the expression, binding activity, and signaling

of the LEPR have been reported (Clément et al., 1998; Farooqi et al., 2007b), mutations that disrupt the downstream signaling cascade have not as yet been clearly associated with obesity. One possible exception is the adaptor molecule, Src homology 2 (SH2) B adaptor protein 1 (SH2B1), which is a key endogenous positive regulator of leptin sensitivity (Maures et al., 2007). However, *SH2B1* mutations have not been shown to disrupt leptin sensitivity, and SH2B1 modulates signaling by a variety of receptor tyrosine kinases, which may explain the additional phenotypes, including severe insulin resistance and behavioral abnormalities, reported in mutation carriers (Doche et al., 2012).

Leptin as a Therapeutic Agent

Recombinant human leptin (metreleptin) is highly effective in patients with no circulating or bioinactive leptin and in those with low endogenous levels with exercise-induced amenorrhea and lipodystrophy. Recombinant leptin has been administered successfully to patients with congenital leptin deficiency for more than 15 years on a named patient basis and was recently approved by the Food and Drug Administration (FDA) for the treatment of generalized lipodystrophy. In contrast, metreleptin has minimal efficacy for more common forms of obesity, which may represent a leptin-tolerant or leptin-resistant state (Heymsfield et al., 1999). In a recent clinical trial, leptin administered in combination with another weight loss agent, pramlintide, a synthetic analog of the pancreatic peptide amylin, had beneficial effects on weight loss, although the precise mechanisms underlying these effects are not entirely clear (Smith et al., 2007). A number of intervention studies have shown that some of the counter-regulatory responses to caloric restriction can be modified by leptin administration, including changes in skeletal muscle and autonomic and neuroendocrine adaptation (Rosenbaum et al., 2002, 2005). This form of intervention could be a useful adjunct in weight-loss maintenance, an area that merits further exploration.

Melanocortin Peptides and Their Receptors

Leptin stimulates primary neurons in the arcuate nucleus of the hypothalamus, which express pro-opiomelanocortin (POMC), which is posttranslationally processed to yield the melanocortin peptides (alpha, beta, and gamma MSH), which are agonists at melanocortin 3 and 4 receptors (Mc3r and Mc4r) expressed on second-order neurons. Leptin signaling modulates energy balance through a combination of melanocortin-dependent/independent pathways. These hypothalamic pathways interact with other brain centers to coordinate energy intake and energy expenditure (Morton et al., 2014).

Several lines of evidence support the critical role of melanocortin signaling in human energy balance. Homozygous null mutations in *POMC* result in severe obesity (Krude et al., 1998), while heterozygous loss-of-function mutations in α- and β-melanocyte-stimulating hormone (α- and β-MSH) significantly increase obesity risk (Biebermann et al., 2006; Lee et al., 2006). Targeted genetic disruption of *Mc4r* in mice leads to increased food intake, increased lean mass, and linear growth (Huszar et al., 1997), phenotypes that overlap entirely with those seen in humans with loss-of-function mutations in *MC4R* (Farooqi et al., 2003). Heterozygous *MC4R* mutations are found in 2%–5% of people with childhood-onset obesity, making this the commonest gene in which highly penetrant variants contribute to obesity (Farooqi et al., 2000; Vaisse et al., 2000). Most disease-causing *MC4R* mutations disrupt the expression and trafficking of MC4R to the cell surface (Lubrano-Berthelier et al., 2006; Xiang et al., 2006). In cells, pharmacological chaperones can increase cell surface expression and signaling of mutant GPCRs, which represents a potentially rational therapeutic approach for this condition (René et al., 2010).

As complete loss-of-function *MC4R* mutations are associated with a more severe form of obesity than partial loss-of-function mutations (Farooqi et al., 2003), modulation of melanocortinergic tone has been the focus of drug development strategies for some time. However, despite promising pre-clinical studies, the first generation of MC4R agonists were small molecules that failed primarily due to safety issues (Van der Ploeg et al., 2002), particularly increases in blood pressure. Loss-of-function *MC4R* mutations are associated with a reduced prevalence of hypertension, low systolic blood pressure, lower urinary noradrenaline excretion, and reduced peripheral nerve sympathetic nervous system activation, revealing that MC4R-expressing neurons represent a key circuit linking changes in weight with changes in blood pressure (Greenfield et al., 2009; Sayk et al., 2010). More recently, a potent melanocortin receptor agonist, RM-493, has been administered as part of a Phase 1B proof-of-concept clinical trial in obese patients, including one cohort of patients with heterozygous loss-of-function mutations in *MC4R*, in whom there was promising weight loss after 4 weeks. If this compound moves forward, this may be one of the first examples of a personalized medicine approach for treating obesity in people with a genetically characterized subtype of obesity.

Processing and Trafficking of Melanocortin Peptides and Receptors

Melanocortin peptides are processed by enzymes including prohormone convertase 1 (PCSK1), which is involved in the cleavage of the precursor peptide POMC into ACTH, which is then further cleaved to generate α-MSH by carboxypeptidase E (Pritchard et al., 2002). Impaired *POMC* processing may contribute to the obesity seen in people with homozygous/compound heterozygous mutations in *PCSK1* who also have glucocorticoid deficiency, hypogonadotropic hypogonadism, and postprandial hypoglycaemia (as a result of impaired processing of proinsulin to insulin) (Jackson et al., 1997; O'Rahilly et al., 1995). Impaired processing of gut-derived peptides may contribute to the neonatal enteropathy seen in PCSK1 deficiency (Jackson et al., 2003; Martín et al., 2013). Intriguingly, common variants that affect the enzymatic activity of PCSK1 have been associated with obesity in multiple European, Asian, and Mexican populations, providing a clear example where both common and rare variants in the same gene can influence a spectrum of variation in body weight (Benzinou et al., 2008; Choquet et al., 2013; Rouskas et al., 2012).

Several human obesity disorders (e.g., Alström syndrome and Bardet-Beidl syndrome) disrupt genes involved in ciliary function (Ansley et al., 2003). The role of neuronal cilia in protein trafficking—in particular, of GPCRs involved in energy homeostasis as well as in leptin signaling (Ainsworth, 2007)—is beginning to

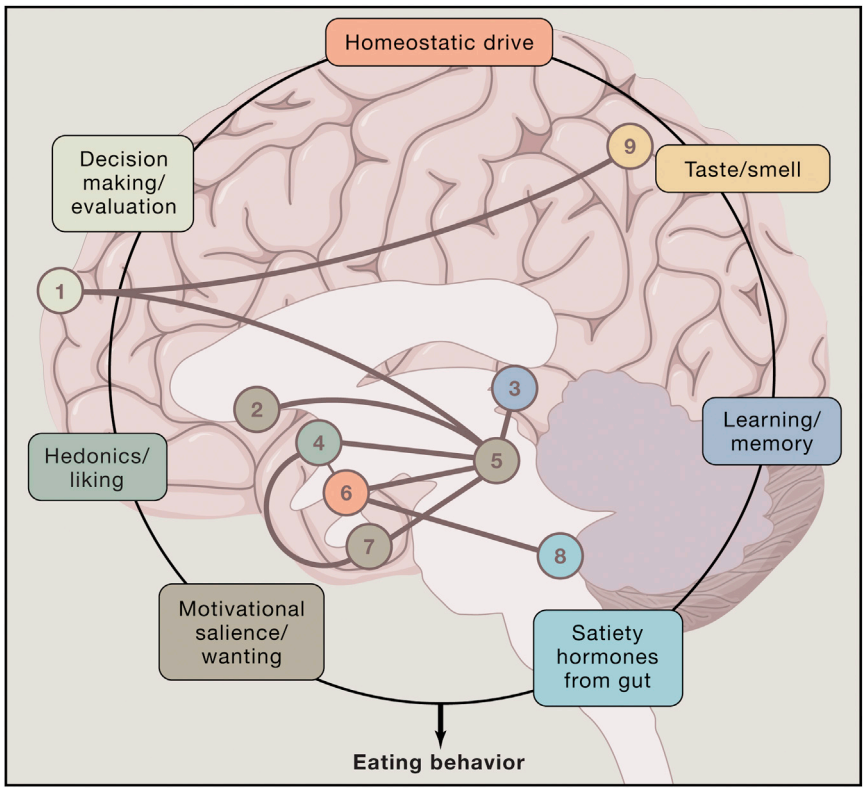

Figure 3. Neural Circuits Involved in Eating Behavior

Neural control of essential behaviors like eating requires the integration of multiple neural signals from different nodes in the brain. Dopaminergic circuits in regions such as the striatum (2), ventral tegmental area (5), and amygdala (7) encode motivational salience and wanting. Opioidergic circuits in regions such as the nucleus accumbens and the ventral pallidum (4) encode hedonic liking. These brain areas and others are integrated with the hypothalamus, cortical areas, and brainstem areas in the regulation of appetite and food intake. Brain regions: (1) prefrontal cortex, (2) dorsal striatum, (3) hippocampus, (4) nucleus accumbens/ventral pallidum, (5) ventral tegmental area, (6) hypothalamus, (7) amygdala, (8) nucleus of solitary tract, (9) gustatory/somatosensory cortex.

emerge. Furthermore, conditional postnatal knockout of proteins involved in intraflagellar transport in neurons and specifically when targeted to *pomc* neurons in mice results in hyperphagia and obesity (Davenport et al., 2007).

Additionally, there is currently a great deal of interest in identifying chaperones and accessory proteins that might modulate melanocortin signaling and melanocortin-dependent pathways. Mrap2, an accessory protein that interacts with Mc4r (and potentially other GPCRs) (Sebag et al., 2013) leads to obesity when disrupted in mice (Asai et al., 2013). Rare variants in *MRAP2* have been associated with severe obesity in humans, although the detailed molecular mechanisms underlying this association are not known (Asai et al., 2013).

Development and Maintenance of Neural Circuits Involved in Eating Behavior

Functional dissection of the neuronal circuits involved in the regulation of energy balance has until recently been limited to dissecting relatively simple linear relationships between neuronal populations that, in reality, are likely to be overlapping and interconnected. Peripheral signals such as leptin can modulate the development and maintenance of these neural circuits (Bouret et al., 2004) and their ability to adapt signaling by altering synaptic inputs (Pinto et al., 2004). While our current understanding of the dynamic and integrated nature of these neuronal networks is still at an early stage, optogenetic tools and other methodologies that permit the manipulation of gene expression in specific populations of neurons are paving the way for major advances in our understanding of the neural circuits connecting brain regions that contribute to the modulation of eating behavior (Betley et al., 2013; Wu et al., 2009) (Figure 3).

Several lines of evidence suggest that brain-derived neurotrophic factor (BDNF), a nerve growth factor that signals via the tyrosine kinase receptor tropomycin-related kinase B (TrkB), is important not only in energy balance, but also in anxiety and aggression. Haplo-insufficient mice and mice in which BDNF has been deleted postnatally are obese with hyperphagia and hyperactivity (Lyons et al., 1999; Xu et al., 2003); this unusual combination of phenotypes is also seen in individuals with genetic disruption of BDNF and TrkB (Gray et al., 2006; Yeo et al., 2004). While a Trkb agonist results in weight loss in mice (Tsao et al., 2008), central administration had no effect on food intake in primates (Perreault et al., 2013). Its potential utility in the treatment of a number of neurodegenerative diseases is still being explored (Yang et al., 2014).

Sim1 is a transcription factor involved in the development of the paraventricular and supraoptic nuclei of the hypothalamus and additionally may mediate signaling downstream of Mc4r (Michaud et al., 1998). *Sim1* haplo-insufficiency in mice and deletions, balanced translocations, and loss-of-function mutations in humans cause severe obesity (Bonnefond et al., 2013; Holder et al., 2000; Ramachandrappa et al., 2013). Oxytocin mRNA levels are reduced in mouse models of *Sim1* deficiency, and oxytocin administration reduces food intake in *Sim1*-haploinsufficient animals (Kublaoui et al., 2008). Impaired oxytocinergic signaling has also been implicated in the hyperphagia and obesity seen in Prader-Willi Syndrome (PWS) (Swaab et al., 1995), caused by lack of expression of a cluster of maternally imprinted snoRNAs on chromosome 15 (Sahoo et al., 2008). People with PWS and with *SIM1* mutations exhibit a spectrum of behavioral abnormalities that overlap with autism-like features and could be related to reduced oxytocinergic signaling (Ramachandrappa et al., 2013), although this has not been tested.

Central administration of oxytocin in rodents is anorexigenic, and rodents that lack oxytocin or the oxytocin receptor become

obese (Olson et al., 1991). The exact sites of action of locally released oxytocin are unknown but likely involve areas with high oxytocin receptor expression, such as the VMH and amygdala. α-MSH, through its effects on MC4R, induces dendritic release of oxytocin, and this locally released oxytocin may be involved in the regulation of appetite (Sabatier et al., 2003). Modulation of central oxytocin signaling therefore forms another potential target in the treatment of obesity (Morton et al., 2014).

Neural Circuits Involved in Eating Behavior

The most consistent phenotype associated with genetic disruption of leptin-melanocortin signaling in humans is hyperphagia, an increased drive to eat (O'Rahilly and Farooqi, 2008). Additionally, detailed characterization of eating behavior in large numbers of twins suggests that eating behavior phenotypes such as satiety responsiveness, eating in the absence of hunger, reinforcing value of food, and the capacity to voluntarily inhibit eating are potentially heritable components of eating behavior (Carnell et al., 2008).This is not surprising, as one of the primary functions of the brain during periods of negative energy balance is to reprioritize behavioral outputs to obtain and consume food, thereby replenishing depleted energy stores. Ensuring sufficient energy stores is critical for survival of the species and, based on our understanding in other mammalian species, multiple processes that defend against starvation and fasting are hardwired.

In addition to this homeostatic regulation of eating behavior, which is driven by energy demands, hedonic food intake (i.e., beyond the need for energy repletion) in response to the rewarding properties of food (Kenny, 2011) is an important contributor to overeating. The palatability of a particular food source is assumed to be related to the flavor and taste of that food; high-fat diets are generally considered more palatable than low-fat diets and are preferentially overconsumed. Neural circuits involving the amygdala, the striatonigral pathway, orbito- and prefrontal cortex, and hippocampus have been implicated in transposing motivational aspects of stimuli into motor responses, as well as in hedonic evaluation of the stimulus and associative learning about the hedonic properties of food (Figure 3). Food reward has been considered to be encoded by distinct neural substrates, opioidergic brain pathways mediating liking (pleasure/palatability), whereas the wanting of food (incentive motivation) appears to be mediated by dopaminergic circuits (Berridge, 1996; Pecina et al., 2003). The overarching role of these responses is to shift attention and effort toward obtaining food reward.

Hormonal regulators of energy homeostasis can also act on brain reward circuits, most notably on the mesoaccumbens dopamine system, to increase or decrease the incentive value of food depending on energy requirements. This suggests that obtaining the pleasurable effects of food is a powerful motivating force that can override homeostatic satiety signals, and in agreement with this, meals that consist of palatable food are generally consumed with greater frequency and in greater portion size than those consisting of less palatable food. As a single meal of increased portion size can trigger increased food intake over several days, such hedonic overeating is likely to be an important contributor to weight gain and the development of obesity.

Human Brain Imaging Studies—Insights into Food Reward

Neural processes such as food reward can be challenging to measure in humans. Imaging studies using functional MRI (fMRI) permit the measurement of blood-oxygen-level dependent (BOLD) signals that reflect neural activity in specific regions involved in the response to food cues (Selvarajah et al., 2014). Pictures of food activate dopaminergic regions such as ventral striatum, and these effects are modulated by homeostatic state (Ziauddeen et al., 2012). In leptin-deficient humans, images of food (compared to non-food images) are associated with a marked increase in neuronal activation in the ventral striatum (Farooqi et al., 2007a). This response was normalized by 7 days of leptin treatment before significant weight loss had occurred, consistent with the view that activation in the ventral striatum does not directly encode the "liking" but, rather, the motivational salience, or "wanting," of food. Studies in obese volunteers in an energy-restricted, partially leptin-deficient state are consistent with the view that these responses are part of the physiological response to energy restriction (Rosenbaum et al., 2008) and are in keeping with findings in experimental studies in rodents (Fulton et al., 2006; Hommel et al., 2006).

Compared to obese controls, obese people with *MC4R* mutations have a preserved pattern of activation of the reward system to visual food cues, suggesting involvement of MC4R in the dopaminergic reward circuitry in humans (van der Klaauw et al., 2014). These findings are supported by evidence in rodents, which suggests that melanocortin signaling modulates food reward. Of note, fMRI studies in Prader-Willi Syndrome have also shown higher neural activity to food cues in reward areas compared to matched obese controls such as accumbens, amygdala, and ventromedial prefrontal cortex (Hinton et al., 2006).

The μ-opioid receptor system that subserves the neural substrates of "liking of food" is a key mediator in the hedonic valuation process of food intake. In addition, μ-opioid receptors were found to mediate the autoinhibition of β-endorphin on hypothalamic *pomc* neurons (Cowley et al., 2003). Antagonism of μ-opioid receptors thus likely results in alterations of hedonic valuation of food as well as potentially attenuates downregulation of *pomc* neuronal activity. Indeed, in humans, the μ-opioid receptor antagonist naloxone reduces the hedonic responses to, and consumption of, palatable foods. In clinical trials, the μ-opioid receptor antagonist GSK1521498 reduces the hedonic response to and motivation for high-fat foods (Ziauddeen et al., 2013). Recently, the combination of naltrexone, an opioid receptor antagonist with high affinity for the μ-opioid receptor, and bupropion, an atypical antidepressant that inhibits reuptake of dopamine and norepinephrine and increases activity of POMC neurons (Contrave) was approved for treatment of obesity by the FDA.

Taste and Food Preference

The orosensory properties of foods are perceived through a combination of taste, texture, and olfaction. The heritability of taste is well established in twin and family studies, with heritability estimates of 30%–50% for pleasantness, consumption, and cravings for sweet foods (Keskitalo et al., 2008). The central

sensing mechanisms for nutrients and quality of food have only recently become the subject of studies. Fat provides twice as many calories per gram as protein or carbohydrate. It is well established that palatable food that is rich in fat and refined sugars promotes larger meal sizes, less postprandial satiety, and greater caloric intake than diets that are high in carbohydrates but low in fat (Salbe et al., 2004). Traditionally, there have been contrasting perspectives on the mechanisms underlying food palatability. The homeostatic view of palatability suggests that palatability reflects the underlying biological need for nutrients, while the hedonic view of palatability suggests that certain foods engage reward processing and are therefore palatable. Studies in rodents have suggested that specific neural pathways, for example, involving the melanocortin-4 receptor (*Mc4r*), play a role in the preference for dietary fat and against dietary sucrose (Panaro and Cone, 2013). To date, very few studies have addressed the preference for specific nutrients in humans, although twin studies have found heritability estimates of 53%–62% for the intake of/preference for foods that are high fat/sucrose. There is considerable research being performed within the food industry focusing on the development of foods that offer some of the sensory properties of fat (fat mimetics) but do not have a high fat content. The potential to modify foods for health benefits is an area of considerable development; such work will need to take into consideration an understanding of the fundamental biology that underpins aspects of eating behavior.

Gut-Derived Satiety Signals

Peptides such as ghrelin, peptide YY (PYY), and glucagon-like peptide 1 (GLP-1) are secreted from gut entero-endocrine cells in response to meal ingestion and the presence of nutrients in the intestinal lumen (Batterham et al., 2002; Turton et al., 1996). Pioneering human infusion studies have demonstrated that a number of gut peptides modulate food intake when administered acutely in humans (Tan and Bloom, 2013), suggesting that modulating satiety signals could be a useful therapeutic strategy in obesity (Finan et al., 2015). The synthetic GLP-I receptor agonist liraglutide has recently been approved for the treatment of obesity alone by the FDA. Several other gut peptide analogs, as well as gut hormone receptor agonists, are currently being studied in clinical trials (Tan and Bloom, 2013).

Satiation, the sensation of fullness that results in meal termination and satiety, the persistence of fullness that determines the timing to the next meal, are heritable traits that influence weight gain (Carnell et al., 2008). Although common obesity seems to be associated with low circulating PYY levels (Batterham et al., 2006), rare genetic variants in PYY or its receptors have not been associated with obesity. Fasting ghrelin levels have been found to be increased in children (Haqq et al., 2003) and adults with PWS (Cummings et al., 2002), potentially contributing to the hyperphagia and impaired satiety associated with this syndrome, although the potential mechanisms involved are not known.

Additionally, there is a growing literature on changes in the composition of the gut microbiome in response to acute/short-term changes in the diet, chronic states such as obesity and bariatric surgery (Turnbaugh et al., 2006), and the impact of specific organisms on nutrient absorption and on metabolic parameters in mice and humans (Cox et al., 2014).

Targeting Energy Expenditure

A number of large family-based population studies, most notably the Quebec family study, have addressed the contribution of genetic versus environmental factors to energy expenditure, including physical activity (Pérusse et al., 1989). For example, the heritability of exercise participation is entirely accounted for by common familial environment, while for physical activity level, the heritability is ~20%. As such, promotion of increased levels of physical activity is a useful strategy for weight loss and, in particular, for weight maintenance.

In contrast, basal metabolic rate (BMR) and respiratory quotient (ratio of carbohydrate versus fat oxidation; a marker of substrate utilization) are highly heritable (47% and 36%, respectively) (Bouchard and Tremblay, 1990). Very few genes have been shown to modulate BMR in humans, although the reduced basal metabolic rate reported in obese people harboring loss-of-function mutations in the cellular scaffolding protein *KSR2* (kinase suppressor of Ras2) suggests that genetic variation in energy expenditure phenotypes may contribute to weight gain in some individuals (Pearce et al., 2013). In this study, almost all of the *KSR2* variants identified in obese individuals impaired glucose oxidation and fatty acid oxidation in cells, suggesting a defect in substrate utilization, which was rescued by the addition of metformin. Further work will be needed to see whether these observations can be replicated in experimental clinical studies and to investigate the cellular mechanisms underlying these effects which, in part, may be mediated by the interaction of KSR2 with the cellular fuel sensor, AMP-kinase (Brommage et al., 2008; Costanzo-Garvey et al., 2009).

The development of compounds that might increase energy expenditure is being explored as a possible therapeutic strategy. One potential route is to activate brown adipose tissue, thereby generating heat through uncoupling protein 1 (UCP1) (Lowell and Spiegelman, 2000). UCP1-positive cells in white adipose tissue depots in rodents (often called beige/brite cells) can be stimulated to dissipate energy by thermogenesis and pharmacological stimulation of these processes, potentially through circulating myokines that drive brown-fat-like development (Wu et al., 1999), has attracted the interest of a number of pharmaceutical companies. Although UCP1-positive cells that show similarity to murine beige adipocytes have been found in human fat depots (Wu et al., 1999), the translation of these findings in rodents to therapies that can be administered safely in humans presents some challenges. For example, what influences the exact amount of brown fat and/or beige fat available in adult humans, and can this be increased? To what extent do sex steroids (or other gender-specific factors) influence the activity/quantity of brown/beige fat, as women seem to have more than men (Cypess et al., 2009)? How much extra energy would be expended through the stimulation/overstimulation of such processes, and would this be clinically relevant? Would an increase in energy expenditure lead to a compensatory increase in food intake, and how might such an effect be managed?

Building an Integrated View of the Pathways that Regulate Energy Homeostasis

Given the complexity of neurobiological processes underlying body weight homeostasis, it is likely that future drugs will need

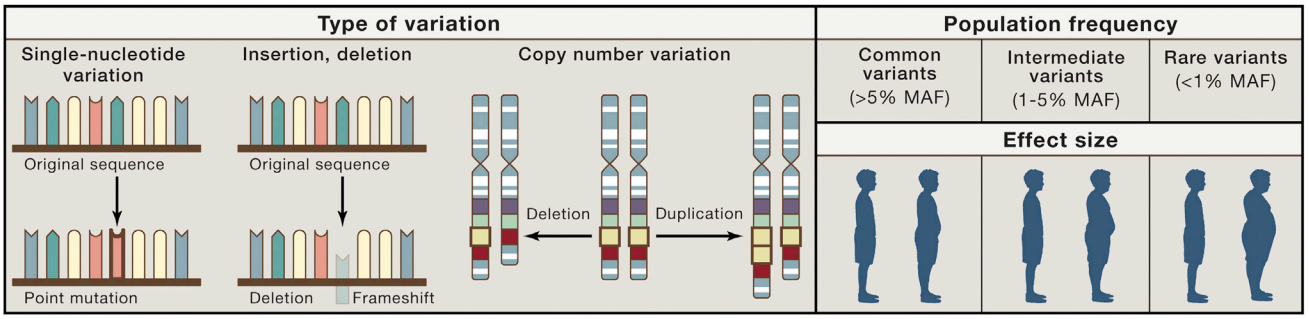

Figure 4. Types of Genetic Variation Contributing to Body Weight Regulation

Genetic effects on body weight are mediated by different types of variants, their frequency in the population, and the effect of the variant on the phenotype. Variants include single-nucleotide variations in which only one nucleotide is changed, copy number variations in which a stretch of DNA is repeated or deleted (often containing many genes), or small insertions and deletions of a few base pairs. Common variants are found at a minor allele frequency (MAF) of more than 5% in a population, whereas intermediate (1%–5%) and rare variants (< 1%) are found at lower frequencies. Generally, the effect size of common obesity-associated variants on body weight is modest. Several rare variants have been associated with severe obesity.

to be directed at highly specific targets and may consist of combinations of compounds that target different mechanisms, as illustrated by recent studies demonstrating the efficacy of dual melanocortin-4 receptor and GLP-1 receptor agonism (Clemmensen et al., 2015). The central and peripheral regulation of food intake, energy expenditure, physical activity, fat absorption, and oxidation are all being explored as potential mechanisms that can be targeted in rodent studies. In parallel, genetic approaches into human eating behavior and obesity may inform the focus of experimental approaches in rodents and might generate new potential drug targets in which the potential relevance to humans may be established at an earlier stage than has previously been the case.

Common Genetic Variants and Genome-wide Association Studies

Genetic influences are likely to operate across the weight spectrum but may be more penetrant when studying childhood-onset obesity and at both extremes of the BMI distribution—thinness and severe obesity. Genetic variance depends on the nature and amount of mutational variance in a population, the segregation and frequency of the alleles that influence a trait in a particular population, the effect sizes of the variants (which may be additive or non-additive), the mode of gene action, and the degree of genetic control of phenotypic variance of the trait in question (Figure 4).

Genome-wide association studies (GWAS) seek to identify the common variants (minor allele frequency [MAF] of more than 5%) that contribute to the heritability of common diseases. High-throughput arrays have facilitated the genotyping of thousands of common variants (directly or by imputation) in large population-based cohorts on whom BMI data is available. The first GWAS-derived loci to be reported were intronic variants in *FTO* (fat mass and obesity associated) and a variant ~200 kb downstream of *MC4R* (Dina et al., 2007; Frayling et al., 2007; Loos et al., 2008). To date, more than 80 genetic loci associated with BMI and body fat distribution (often measured by waist-to-hip ratio) have been identified by GWAS approaches, and many of these have been replicated in different populations and ethnicities (Locke et al., 2015). GWAS in childhood-onset obesity and in severely obese children and adults have shown that there is some overlap between the common variants that contribute to early-onset and adult-onset weight gain, but also that both of these approaches can identify novel variants (Bradfield et al., 2012; Wheeler et al., 2013). Cumulatively, the common variants identified in GWAS are characterized by modest effect sizes (per allele odds ratios between 1.1 and 1.5), and the proportion of variability of BMI explained by GWAS-identified loci to date remains relatively modest (< 5%). Nevertheless, variants that explain a small proportion of phenotypic variance may provide substantial biological or therapeutic insights, although the road to establishing causal variants and their functional relevance is often a challenging one.

GWAS-associated loci are often identified by the name of the nearest gene; this may or may not be the gene in which variation contributes to variation in BMI. Some of the GWAS loci encompass genes previously appreciated to play a role in energy homeostasis (e.g., *LEPR*, *SH2B1*, *MC4R*, *BDNF*), and in some cases, specific variants have been associated with changes in expression based on eQTL data (Wheeler et al., 2013). Other loci contain genes that seem to be plausible biological candidates or can suggest genes for which there was no previous evidence (Locke et al., 2015). Many of the signals identified to date map to non-coding regions of the genome that may potentially be involved in gene regulation.

The strongest association signal for BMI has consistently been found with variants in the first intron of FTO, which have been associated with increased BMI and eating behavior in a number of studies (Cecil et al., 2008; Wardle et al., 2008a). Deletion or overexpression of *fto* and other genes in this region (*IRX3*, *RPGRIP1L*) in rodents (Church et al., 2010; Fischer et al., 2009; Gerken et al., 2007; Stratigopoulos et al., 2008) (Smemo et al., 2014) can impact energy homeostasis. Despite these obvious challenges, these studies have demonstrated progress toward identifying new biology based on GWAS (Tung et al., 2014).

Is there yet more common variation to find? Newly developed statistical methods that assess the contribution of common genetic variation across the genome (Zhu et al., 2015) support the growing consensus that there is a long tail of common

variation. As such, meta-analyses of even larger population-based data sets are currently underway. The available evidence suggests that BMI is highly polygenic (high number of contributing genes) (Gusev et al., 2014). One of the challenges of such studies is how to capture the full spectrum of genetic variation (Figure 4), including complex multi-allelic CNVs, which show lower linkage disequilibrium with surrounding SNPs and are consequently less detectable by conventional SNP-based genome-wide association studies. For example, in a large family-based association study of Swedish families ascertained through the identification of siblings who were discordant for obesity, integrating data from CNV analysis with transcriptomic data from adipose tissue revealed an association with copies of *AMY1* with obesity (Falchi et al., 2014).

Finding New Rare Highly Penetrant Variants

Rare variants, which outnumber common variants in the human genome, may explain a proportion of the heritability of obesity and may be more readily identified at the extremes of the phenotypic distribution. The earliest studies were performed in children with clinically identifiable syndromes often associated with developmental delay or dysmorphic features as well as obesity. Rare CNVs that often disrupt a number of genes have recently been implicated in highly penetrant forms of obesity (Bochukova et al., 2010; Walters et al., 2010). Candidate gene studies based on the molecules known to cause severe obesity in experimental animals have shown that these genes also contribute to childhood-onset human obesity, often in the absence of developmental delay. The functional and physiological characterization of these mutations and of the mutation carriers has illustrated a high degree of convergence of the mechanisms that regulate energy balance across mammalian species.

Exome sequencing of cohorts with severe childhood-onset and adult-onset obesity, as well as those at the extremes of the BMI distribution in population-based cohorts, is well underway and may lead to the identification of new genes whose functions will need to be explored in cells, model organisms, and humans. Whole-genome sequencing provides the ''most complete'' view of genomic variation but poses challenges in terms of proving causality, but these are beginning to be addressed. Recent studies have now shown that human inducible pluripotent stem cell (iPSC)-derived neurons may facilitate a mechanistic understanding of how specific genes disrupt cellular and neuronal mechanisms that may be involved in the pathogenesis of obesity (Wang et al., 2015).

Therapeutics Opportunities in Obesity

Lifestyle modification remains the first step in weight management. While intervention programs that focus on supporting people to change their diet and/or levels of physical activity can be effective in inducing weight loss in the short to medium term in some people, they lose efficacy in the long term. As such, in addition to the focus on prevention of obesity, treatment of obese patients, preferably at a stage before complications have emerged, is an important priority (Gray et al., 2012). However, current therapeutic options in obesity are very limited; the only currently approved anti-obesity drug for long-term use in the US and Europe is Orlistat, which reduces intestinal lipid absorption by inhibiting pancreatic lipase and often has limiting adverse effects that preclude its long term use.

Previously available anti-obesity drugs targeted cannabinoid signaling (rimonabant), noradrenergic (phentermine) and serotoninergic signaling (fenfluramine, dexfenfluramine), and reuptake (sibutramine). These compounds were moderately effective but, as with many centrally acting agents, at the expense of many off-target effects, reflecting lack of specificity of the neural targets. Lorcaserin, a selective 5HT2cR agonist with limited activity at the other serotonin receptors, has been approved for use in the US (Smith et al., 2010), although concerns about potential cardiac valvulopathy and cancer risk have prevented European approval of the drug to date. The combination of the anticonvulsant topiramate and phentermine, which increases central noradrenaline levels (Qsymia), is also approved in some countries.

Finally, development of personalized medicine by selecting the optimal pharmacological intervention for particular people through genetics or other molecular/cellular analyses is an exciting and evolving area. Synthetic-biology-inspired therapeutic systems that integrate sensor and effector devices into cells have been developed to monitor disease-relevant metabolites, process on/off level control, and coordinate adjusted therapeutic responses. These systems have the potential to restore metabolite homeostasis in a seamless, automatic, and self-sufficient manner, which is particularly attractive for future gene- and cell-based therapies. As an example, a closed-loop synthetic intracellular lipid-sensing receptor (LSR)-pramlintide circuit represents a potential prototype for such a cell-based therapy. The LSR sensor captures a wide range of lipids within their physiologic concentration range, becomes dose-dependently activated by peak fatty acid levels, and is turned off at physiological concentrations (Rössger et al., 2013). Such emerging methodologies offer fresh perspectives for drug delivery and potentially personalized medicine in the future.

ACKNOWLEDGMENTS

We would like to thank the many colleagues, collaborators, and referring Physicians with whom we have worked over the years. We thank the Wellcome Trust, MRC, ERC, NIHR Cambridge Biomedical Research Centre, and Bernard Wolfe endowment for their support for this work. Importantly, we would like to thank the patients and their families for their contributions. Further information can be found at http://www.goos.org.uk.

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Leading Edge
Review

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Neural Control of Energy Balance: Translating Circuits to Therapies

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http://dx.doi.org/10.1016/j.cell.2015.02.023

Recent insights into the neural circuits controlling energy balance and glucose homeostasis have rekindled the hope for development of novel treatments for obesity and diabetes. However, many therapies contribute relatively modest beneficial gains with accompanying side effects, and the mechanisms of action for other interventions remain undefined. This Review summarizes current knowledge linking the neural circuits regulating energy and glucose balance with current and potential pharmacotherapeutic and surgical interventions for the treatment of obesity and diabetes.

Introduction

Obesity, diabetes, and associated disorders represent a major public health challenge for North America, Europe, and increasingly the rest of the world. Both obesity and diabetes inflict health and economic burdens that require coordinated strategies to both prevent and treat these disorders. Indeed, a major barrier in the management and prevention of obesity is that weight loss due to lifestyle changes alone is inherently difficult. For many, this means that dieting-induced weight loss initially results in tangible beneficial effects but is often followed by a return to previous energy intake and consequently a rebound weight gain.

Numerous neurobiological and physiological mechanisms that regulate energy balance exist. In particular, it has become increasingly evident that the brain plays an important role in sensing energy demands and storage in order to maintain/defend body weight within a rather tight range. Studies ranging from worms, flies, and mice to humans have identified key conserved genes and neural pathways that are critical in regulating energy balance and glucose homeostasis. Moreover, the identification of human mutations in these or analogous pathways has led to hope that it may be possible to develop rational strategies based on animal model studies that may ultimately lead to successful therapeutic intervention in humans. In this Review, we will highlight how advances in understanding the neurophysiology underlying metabolism, including an increased understanding of neural circuits, may hold promise for development of adjunct therapies in the treatment of obesity and associated co-morbidities, including diabetes. Several recent Reviews have provided more detailed information and review of the primary literature regarding the respective circuits and approaches highlighted here (Barsh et al., 2000; Cone, 2005; Deisseroth, 2012; Farooqi and O'Rahilly, 2005; Heisler et al., 2003; Myers and Olson, 2012; Powley et al., 2005; Schwartz and Porte, 2005; Wikberg and Mutulis, 2008).

A Brief Overview of Neural Circuits Regulating Feeding and Energy and Glucose Homeostasis

The central melanocortin system is comprised of neurons in the hypothalamic arcuate nucleus and brainstem that produce pro-opiomelanocortin (Pomc), the precursor polypeptide of the biologically active melanocortin receptor peptide agonist, α-melanocyte-stimulating hormone (α-MSH). Additional peptides within the arcuate nucleus that contribute to the melanocortin system include Agouti gene-related peptide (AgRP), an endogenous inverse agonist of the melanocortin 4 receptor (Mc4r), and Neuropeptide Y (NPY), which is co-expressed with AgRP. Elucidating the physiological importance of this system in regulating energy balance and glucose homeostasis brought the hypothalamic arcuate nucleus to the forefront of research aimed at understanding the neural control of energy balance (Cone, 2005; Schwartz and Porte, 2005).

Pomc and NPY/AgRP neurons are prototypical players in the regulation of energy intake and expenditure for several reasons. In particular, exogenous administration of α-MSH potently inhibits food intake via activation of central melanocortin receptor-expressing neurons (Cone, 2005; Rossi et al., 1998; Schwartz and Porte, 2005). Conversely, administration of NPY effectively stimulates food intake via action at NPY-Y receptors in the brain (Clark et al., 1984; Yulyaningsih et al., 2011). Several studies have used opto- and chemogenetic techniques to attempt to manipulate the activity of varying genetically targeted populations of neurons with a role in feeding behavior and metabolism, including but not limited to AgRP neurons (Aponte et al., 2011; Atasoy et al., 2012; Krashes et al., 2011; Krashes et al., 2013) and Pomc neurons (Aponte et al., 2010; Zhan et al., 2013). Stimulation of arcuate Pomc neurons resulted in a reduction in food intake, whereas activation of arcuate AgRP neurons resulted in increased food intake and food-seeking behaviors (Aponte et al., 2010; Krashes et al., 2011; Zhan et al., 2013). The Pomc-induced reduction in food intake was

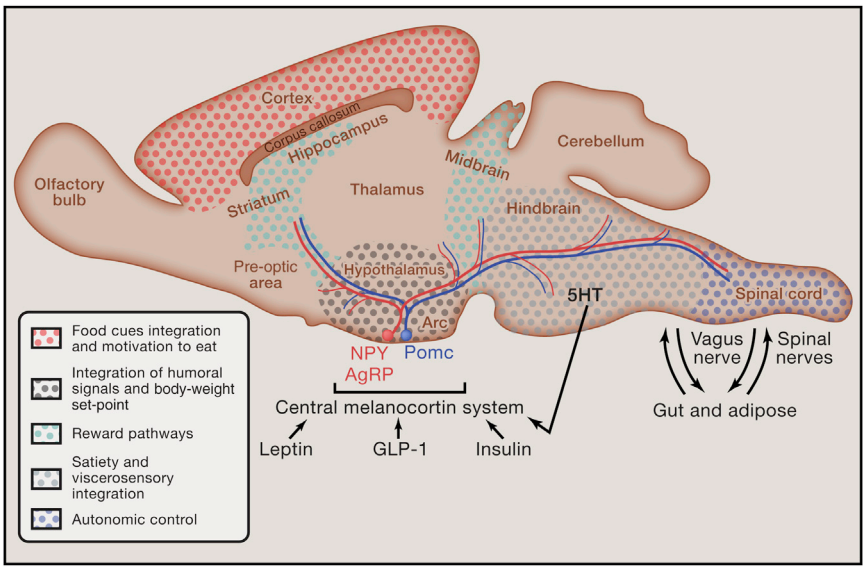

Figure 1. Integrated Model of the Central Melanocortin System and Connected Regions within the Nervous System Involved in Obesity and Diabetes

Recent discoveries highlight a circuitry within the brain that includes the hypothalamus as well as midbrain/hindbrain areas in the regulation of energy expenditure. This circuitry has also been demonstrated to have an overlapping role in the management of glucose homeostasis. Importantly, several neurotransmitters and peptides contribute to a distributed network of receptor systems within this circuit and provide potential substrates for non-surgical therapeutic intervention. Similarly, advancing technologies have raised potential surgical and non-surgical manipulation of cellular and nerve activity as a viable strategy in this circuit to combat obesity and diabetes. Abbreviations: NPY = neuropeptide Y; Pomc = pro-opiomelanocortin; AgRP = Agouti gene-related peptide; Arc = arcuate nucleus; GLP-1 = glucagon-like peptide 1.

dependent upon melanocortin receptors within the paraventricular hypothalamus (PVH), a hypothalamic nucleus that is a direct target of arcuate melanocortin neurons. Stimulation of arcuate AgRP neurons elicited feeding behavior via projections to the thalamus, the hypothalamus, and the basal forebrain (Atasoy et al., 2012). Interestingly, either the neurotransmitter GABA or NPY is required for the rapid stimulation of feeding, whereas the neuropeptide AgRP, through action on Mc4 receptors, is sufficient to induce feeding over a delayed yet prolonged period (Krashes et al., 2013). GABA from NPY/AgRP neurons of the hypothalamic arcuate nucleus may also play an important role in the regulation of feeding behaviors via direct actions within the hindbrain (Wu et al., 2009). Moreover, neurons of the nucleus of the solitary tract in the caudal medulla might counterbalance this activity (Wu et al., 2012). Neurons of the PVH also reciprocally innervate arcuate NPY/AgRP neurons, providing a regulatory loop in the control of feeding behavior (Krashes et al., 2014). Collectively, these data suggest that in addition to a core circuit, AgRP neuron projections and reciprocal innervations may reveal key modulatory circuit nodes that are gated or otherwise regulated by AgRP neuron projections (Atasoy et al., 2012). Importantly, it's currently unclear which circuits are acutely involved in other complex metabolic processes such as energy expenditure and glucose metabolism. Undoubtedly, similar parallel and redundant mechanisms involved in regulating feeding behavior will be intertwined with various aspects of metabolism.

Both NPY/AgRP and Pomc neurons are also sensitive to metabolic status (Cone, 2005; Yulyaningsih et al., 2011). In particular, NPY/AgRP neurons are activated during fasting, whereas Pomc neurons are activated following feeding and inhibited during fasting. NPY/AgRP and Pomc neurons are also well positioned to sense and integrate numerous nutrient and humoral signals (Schwartz and Porte, 2005). Indeed, many of these signals in the periphery and/or the central nervous system (CNS) have been attributed to regulating feeding behavior and energy expenditure via activity at these neurons (Figure 1). For example, increased serum levels of the adipocyte-derived anorexigenic peptide leptin activate Pomc neurons and stimulate the production as well as the release of α-MSH (Cone, 2005; Schwartz et al., 1997). Leptin inhibits NPY/AgRP neurons concomitantly suppressing the production and release of NPY and AgRP. These data and others support a primary role of arcuate Pomc and NPY/AgRP neurons as first-order neurons in the neural control of energy balance. However, it must be noted that leptin-sensitive neurons distributed across the brain may contribute to the control of feeding. For instance, loss of leptin receptors in either arcuate Pomc neurons or adjacent neurons of the ventromedial hypothalamus (VMH) results in modest increases in adiposity largely dependent upon deficits in energy expenditure (Myers and Olson, 2012). Moreover, deletion of leptin receptors in both Pomc and VMH neurons resulted in an additive effect on body weight. Among other examples, leptin receptors on GABAergic neurons of the lateral hypothalamic area (LHA) modulate the mesolimbic dopaminergic system and decrease feeding (Leinninger et al., 2009). Also, deficiency of leptin receptors in a brainstem site called the dorsal vagal complex causes hyperphagia, resulting in modest weight gain (Scott et al., 2011; Skibicka and Grill, 2009). Collectively, these data support a model of a distributed network of melanocortin- and leptin-responsive neurons contributing to the central regulation of energy homeostasis. Not surprisingly, numerous nutrient and humoral receptors are also widely distributed within the CNS, and researchers are identifying multiple divergent and redundant roles for these receptors in the regulation of metabolism.

The aforementioned evidence supports a well-defined action of the central melanocortin system in regulating food intake and body weight. However, it is less appreciated that the central melanocortin system plays an important role in regulating glucose metabolism, including insulin release and action (Farooqi and O'Rahilly, 2005; Schwartz and Porte, 2005). In fact, deficits in melanocortin signaling result in hyperinsulinemia,

fasting hyperglycemia, and frank diabetes (Berglund et al., 2014; Cone, 2005; Myers and Olson, 2012). However, restoring Mc4r expression selectively in the PVH reduces hyperphagia and largely restores deficits in body weight independent of improved glucose or insulin intolerance (Myers and Olson, 2012). In parallel, loss of Mc4rs in parasympathetic and sympathetic nuclei of the brainstem and spinal cord results in glucose and insulin intolerance largely independent of changes in food intake or bodyweight gain (Berglund et al., 2014; Myers and Olson, 2012). Thus, the wide distribution of melanocortin receptors concomitant with projection patterns of Pomc and NPY/AgRP neurons contributes to their divergent effects on energy and glucose homeostasis.

Several mutations in Mc4r have been identified and may represent the most common form of monogenic obesity in humans (Barsh et al., 2000; Farooqi and O'Rahilly, 2005). Importantly, the melanocortin system is remarkably conserved across species from zebrafish and rodents to humans (Farooqi and O'Rahilly, 2005; Sebag et al., 2013). Thus, neural circuits regulating metabolic homeostasis in the mammalian hypothalamus have an ancient origin, dating back to teleost fish. This long evolution may explain the complexity as well as some of the main anatomical and functional principles of these circuits governing energy homeostasis in the mammalian hypothalamus.

In Search of Effective Appetite Suppressants

Although dieting and balanced nutrition are key to the management of most, if not all, metabolic disorders, most dieters struggle to maintain a rigid dietary regimen. Why is dieting so difficult? There is certainly no single straightforward answer to this question; however, brain-imaging studies in humans have offered clues to neural correlates of obesity and responses to dieting. In particular, dieting in humans changes the activity of interconnected brain regions involved in the neural representations of hunger and satiety and the anticipation of reward (Rosenbaum et al., 2008). Neural activity induced by food cues also differs between obese and lean individuals in the prefrontal cortex (Zheng and Berthoud, 2008). Paradoxically, these regions are involved in the motivation to eat and are normally stimulated by dieting and starvation. Typically, the brains of obese individuals appear to react to food cues as if these individuals were in a state of negative energy balance (instead of energy repletion). Put another way, defective brain processing in response to food cues in obesity may result in inadequate reward and/or motivational processes and, ultimately, hyperphagia.

Driven by various biological and environmental factors, hyperphagia is largely responsible for the current epidemic of obesity and type 2 diabetes (T2DM). Thus, any successful weight-loss strategy must address the issue of appetite. Accordingly, many drugs approved by the US Food and Drug Administration (FDA) for the treatment of obesity directly act on the nervous system to suppress appetite (Table 1 and Figure 2) (Vetter et al., 2010). The only peripherally acting drug currently approved to treat obesity is Orlistat (trade name: Xenical or Alli), an inhibitor of lipase activity that prevents fatty-acid absorption from the diet (Bray, 2014). However, this drug commonly promotes loose stools and, due to its lack of effect on the brain, minimally reduces appetite. As the field continues to build an understanding of neural circuitry regulating appetite, it has become evident that a very large number of neural pathways and molecules are implicated in modulating appetite levels. Consequently, the neural circuits involved in metabolism offer numerous avenues for therapeutic intervention in the treatment of hyperphagia (Figure 2). Below we include a brief discussion on current appetite suppressants and promising pharmacotherapeutics, with a special emphasis on their potential mechanisms of action (Table 1 and Figure 2). Information on the clinical features and medical recommendations for the drugs presented below can be found elsewhere (Bray, 2014).

Overview of Current Anti-Obesity Drugs

Serotoninergic neurons play critical roles in the suppression of feeding (Heisler et al., 2003). Accordingly, treatments that suppress central serotoninergic signaling result in hyperphagia and weight gain in humans and rodents. Lorcaserin (trade name: Belviq), an agonist of the serotonin receptor 5-HT_{2C}R, reduces body weight largely dependent upon lowered food intake in both humans and rodents (Martin et al., 2011; Smith et al., 2010; Thomsen et al., 2008). Although 5-HT_{2C}Rs are widely expressed across the neuraxis (Julius et al., 1988), accumulating evidence in rodents indicates that 5-HT_{2C}R reduces appetite and body weight by acting on Pomc neurons (Xu et al., 2008, 2010b). Notably, Lorcaserin is currently the only FDA-approved drug monotherapy for obesity that specifically targets the brain. However, clinical trials indicate that Lorcaserin yields weight losses of 5%–10% of initial body weight after 1 year (Smith et al., 2010) and is not marketed in Europe.

The naturally occurring peptide glucagon-like peptide 1 (GLP-1) induces multiple desirable anti-diabetic and anti-obesity actions, and protease-resistant long-acting GLP-1 analogs are currently available for the treatment of T2DM (Drucker, 2006). Initial interest in the beneficial effects of GLP-1 on body weight stemmed from the observation that GLP-1 inhibits food intake (Turton et al., 1996). Both Exenatide (trade name: Byetta or Bydureon—a synthetically derived exendin-4) and Liraglutide (trade name: Victoza—a fatty acid-modified GLP-1) are long-acting GLP-1 analogs that effectively lower blood glucose in diabetic patients and are approved by the FDA (see Table 1) to treat T2DM (Juhl et al., 2002; Madsbad et al., 2004). Recent evidence further suggests that both Exenatide and Liraglutide cross the blood-brain barrier (Drucker, 2006; Hunter and Hölscher, 2012) and specifically act at the level of the hypothalamus to reduce appetite (Drucker, 2006; Secher et al., 2014; Sisley et al., 2014). Importantly, the FDA recently approved Liraglutide for the treatment of chronic weight management. In particular, Liraglutide results in sustained weigh loss of 5%–10% of initial body weight in obese patients (Astrup et al., 2009), which can be attributed to both reduced appetite (concomitant with inhibition of gastric emptying) and elevated energy expenditure in T2DM individuals (Horowitz et al., 2012). While Liraglutide is a well-tolerated drug, its long-term side effects are not well known, and some concern has been raised due to a possible risk of pancreatic cancer associated with GLP-1 agonist treatment (Cohen, 2013). Moreover, GLP-1 receptor agonists increase heart rate and blood pressure in rats by stimulating the sympathetic outflow to the cardiovascular system (Yamamoto et al., 2002).

Table 1. Select List of Current and Potential Future Therapeutics for the Treatment of Obesity and Diabetes

Pharmacology

Drug/Compound	Trade/Brand Name	Receptors/Molecular Pathways	FDA Approval	Applications(s)	Brain Area and/or Tissues Involved
Lorcaserin	Belviq	5ht2cr agonist	yes—2012	obesity	hypothalamus, cortex, midbrain, and brainstem
Liraglutide	Victoza	GLP-1r agonist	yes—2012	obesity and diabetes	hypothalamus
Topiramate/ Phentermine	Qysemia	inhibit several ionic conductances as well as carbonic anhydrase isozymes/stimulate the release of serotonin-norepinephrine-dopamine	yes—2012	obesity	hypothalamus and brainstem
Buproprion/ Naltrexone	Contrave	dopamine and norepinephrine reuptake inhibitor/opioid receptor antagonist	yes—2014	obesity	hypothalamus
D-fenfluramine	Pondimin, Ponderax, Adifax	5ht2cr agonist	withdrawn	obesity	hypothalamus and brainstem
Orlistat	Xenical and Alli	inhibit gastric and pancreatic lipases	yes—1999	obesity	gut
Sibutramine	Meridia	serotonin-norepinephrine-dopamine reuptake inhibitor	withdrawn	obesity	hypothalamus and brainstem
Ribonamant	Zimulti	CB1R antagonist	withdrawn	obesity	hypothalamus and brainstem
Mc4R agonists	N/A	Mc4R	no	obesity	hypothalamus and brainstem
Exenatide	Byetta, Bydureon	GLP-1r agonist	yes —2005	T2DM	hypothalamus
Metformin	Glucophage, Glumetza, Glucophage XR, Fortamet	suppress gluconeogenesis pathways	yes—1995	T2DM	liver
Pramlintide	Symlin	amylin receptor agonist	yes—2005	T2DM	hypothalamus and brainstem
Leptin	Metreleptin	LepRB agonist	no[b]	type 1 and 2 diabetes	hypothalamus

Device-Assisted

Device	Receptors/Molecular Pathways	FDA Approval	Application(s)	Brain Area and/or Tissues Involved
hfDBS	neuronal excitability	no[a]	obesity	hypothalamus
tDCs	neuronal excitability	no[a]	obesity	cortex
VNS	neuronal excitability	no[a]	obesity	cervical vagus nerve
VBLOC	neuronal excitability	yes—2015	obesity	gastric vagus nerve
Lap-band—laparoscopic adjustable gastric band	unknown	yes—2001	obesity	gut-brain communication
Roux-en-Y gastric bypass	unknown	N/A	obesity and T2DM	gut-brain communication
Vertical sleeve gastrectomy	unknown	N/A	obesity and T2DM	gut-brain communication

[a]Approved for indications not related to metabolic outcomes.
[b]Approved for the metabolic disorders of lipodystrophies.

Current monotherapeutic approaches in the treatment of obesity and diabetes have not been optimally effective. Emerging strategies include combination drug therapy targeting multiple signaling mechanisms. Combination therapies have been more efficacious than the aforementioned monotherapies, presumably due to the additive and synergic effects previously observed by the drugs separately. There are two combination drug therapies currently marketed in the treatment of obesity, Phentermine/Topiramate (trade name: Qysemia) and Bupropion/Naltrexone (trade name: Contrave). All four compounds have been previously approved on an individual basis by the FDA in the treatment of various neurological disorders. Phentermine and Bupropion are well-known stimulators of serotonin and norepinephrine release from nerve endings. The enhanced release of norepinephrine in sympathetically innervated tissues is likely to increase metabolism (Nedergaard and Cannon, 2014). Moreover, at high doses, these two drugs effectively reduce food intake and body weight in laboratory rodents (Roth et al., 2008; Wright and Rodgers, 2013). Topiramate is an anti-convulsant anecdotally reported to induce weight loss

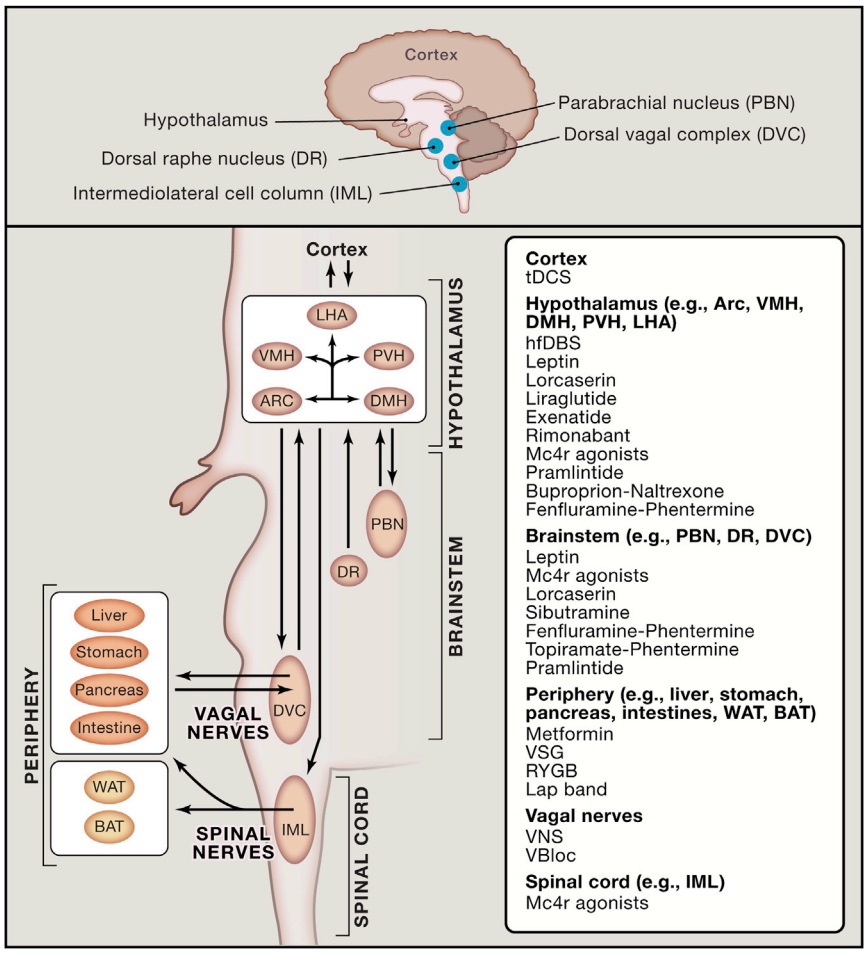

Figure 2. Selected Therapeutic Options for Treating Obesity and Diabetes by Targeting the Brain

Current and promising therapeutics in the treatment of both obesity and diabetes have targeted neural connections including the melanocortin system, via pharmacological and/or device-assisted methods. Here we summarize a select number of targets (i.e., receptors and brain regions) that have been demonstrated to regulate at least in part effects of energy balance and glucose homeostasis.

Abbreviations: hfDBS = high-frequency deep brain stimulation; VBLOC = vagal blocking; VNS = vagal nerve stimulation; Mc4r = melanocortin 4 receptor; DVC = dorsal vagal complex; PBN = parabrachial nucleus; tDCS = transcranial direct current stimulation; Arc = arcuate nucleus; VMH = ventromedial hypothalamic nucleus; LHA = lateral hypothalamic area; DMH = dorsal medial hypothalamic nucleus; PVH = paraventricular hypothalamus; DVC = dorsal vagal complex; IML = intermediolateral cell column; BAT = brown adipose tissue; WAT = white adipose tissue; DR = dorsal raphe nucleus.

(Astrup et al., 2004). Naltrexone is an antagonist of opioid receptors with little effect on food intake when administered alone (Greenway et al., 2009). Although Naltrexone may act by increasing the firing rates of Pomc neurons (Greenway et al., 2009), the exact mechanisms of action of Naltrexone and Topiramate on energy balance are unclear. Importantly, the FDA has recently approved the drug combinations Contrave and Qysemia as an adjunct for chronic weight management (Table 1) with both medications promoting weight losses of 8% to 10% of initial body weight (Allison et al., 2012; Garvey et al., 2012; Greenway et al., 2009). Interestingly, another combination medication called Empatic (trade name) that contains both Bupropion and Zonisamide (another anti-convulsant) is currently under phase 2 clinical trial (Jackson et al., 2014). Thus, with the recent FDA approval and/or promising clinical trial results, combination therapies show promise for therapeutic efficacy in the treatment of obesity.

Novel Paradigms in Anti-Obesity Pharmacotherapy

Although many compounds acting on neural pathways controlling appetite, glucose metabolism, and energy expenditure were found to induce marked body-weight loss in animal models and human clinical trials, leading to FDA approval, many of these pharmacotherapies were subsequently abandoned due to serious safety concerns (Bray, 2009) (Table 1). This was the case for D-fenfluramine (D-fen; trade name: Pondimin, Ponderax, or Adifax), a pharmacological agent that increases serotonin content by stimulating synaptic release of serotonin and blocking its reuptake into presynaptic terminals (Connolly et al., 1997). Although D-fen showed potent anorexigenic activity in humans (McGuirk et al., 1991), it was removed from the market in 1997 due to its link to the development of cardiovascular complications (Connolly et al., 1997). Other centrally acting drugs have succumbed to a similar fate, including Rimonabant (trade name: Zimulti), an antagonist of the type I cannabinoid receptor Cb1R. Rimonabant's anti-obesity actions have been attributed to altered excitability of Cb1Rs located in key hypothalamic, striatal, and brainstem neurons (Cota et al., 2006). Although Rimonabant was highly effective in reducing food intake and body weight, its development was halted in 2009 due to its serious gastrointestinal and adverse psychiatric effects. Although combinatorial approaches have already raised hope for safer ways of treating obese patients, even currently FDA-approved drugs are not without safety concerns. Another caveat of currently available drugs is their modest efficacy when compared to bariatric surgery (Colquitt et al., 2014). The issues discussed above have stimulated research aimed at identifying new classes of centrally acting appetite suppressants, some of which are presented below.

Mc4 receptors have been considered prime targets in the search for effective therapeutics managing obesity (Van der Ploeg et al., 2006; Wikberg and Mutulis, 2008). In fact, several Mc4 receptor agonists were reported to produce significant reduction in adiposity in humans and monkeys (Fehm et al.,

2001; Kievit et al., 2013; Wellhöner et al., 2012). However, Mc4 receptor agonists have not progressed to the clinic due to potential side effects, in particular those affecting the autonomic nervous system. For example, Mc4 receptor agonists such as melanotan II may result in hypertension and priapism (Greenfield et al., 2009; Van der Ploeg et al., 2002). These undesirable effects are likely caused by the stimulation of preganglionic autonomic neurons expressing Mc4 receptors (Sohn et al., 2013). However, all Mc4 receptor agonists may not similarly alter autonomic function. A small peptide, RM-493, has been demonstrated to effectively induce weight loss in non-human primates and humans with no major impact on cardiovascular function (Kievit et al., 2013). This agent is currently in clinical trials for genetic obesity. Interestingly, Mc4 receptors may also be modulated by the melanocortin receptor accessory proteins (Mrap1 and Mrap2) (Asai et al., 2013; Sebag et al., 2013). These Mraps may provide a substrate to differentially target the beneficial effects of Mc4 receptor activity on energy expenditure, glucose utilization, and other metabolic parameters while minimizing the adverse side effects. Recent work has also highlighted a potential role for the melanocortin system to regulate brown adipose tissue thermogenesis as well as the browning/beiging of white adipose tissues (Berglund et al., 2014; Dodd et al., 2015; Nedergaard and Cannon, 2014; Ruan et al., 2014; Williams et al., 2014). It's currently unclear what contribution this thermogenesis may play in the regulation of body composition and glucose homeostasis; however, these data may aid in the development of Mc4 receptor agonists to combat obesity and diabetes.

Recent efforts have also focused on exploring new ways of potentiating the efficacy of currently available appetite suppressants, including, most notably, leptin. Shortly after its discovery (Zhang et al., 1994), leptin was shown to completely reverse the hyperphagic and obese phenotype of leptin-deficient animals and humans (Farooqi and O'Rahilly, 2005; Licinio et al., 2004; Pelleymounter et al., 1995). Unfortunately, obesity is characterized by hyperleptinemia (Considine and Caro, 1996), rather than leptin deficiency. Thus, to the vast majority of morbidly obese individuals, leptin monotherapy is ineffective in lowering food intake or body weight. However, since the normal fall of leptin that accompanies weight loss is detected by the brain as a starvation signal (Ahima and Flier, 2000), it has been proposed that leptin administration during the course of dieting may amplify weight loss and reinforce compliance (Rosenbaum et al., 2008).

Several gut peptides have been demonstrated to potently enhance or restore leptin sensitivity in diet-induced obesity. Most notably, amylin, a pancreatic-derived small peptide, has been shown to successfully enhance the effects of leptin administration on energy balance in obese rodents as well as in humans (Ravussin et al., 2009; Trevaskis et al., 2008). In addition, recent advances in the synthesis of peptides with co-agonistic properties have permitted the development of a new generation of anti-obesity molecules. This includes a GLP-1::glucagon unimolecular co-agonist (Day et al., 2009). This molecule was shown to effectively reduce body weight in obese animals to almost 30% of initial body weight in only 1 month. Leptin-induced body-weight loss has also been shown to be considerably potentiated by the GLP-1::glucagon co-agonist (Clemmensen et al., 2014). The chronic co-administration of leptin and GLP-1::glucagon yielded an impressive 50% weight loss in obese mice over 1 month and normalized glucose intolerance.

Another promising co-agonistic (dual peptide) strategy consists of delivering peptide agonists with an attached (linker) small molecule. This strategy allows for the selective delivery of a complex molecule to particular target cells (Finan et al., 2012). In the first usage of this strategy, researchers capitalized on the mono-agonistic properties of GLP-1 and the sex steroid hormone, estrogen, to improve metabolic parameters of obesity and T2DM. A fully active GLP-1 agonist stably linked to estrogen consistently proved to be more efficacious in lowering body weight than either molecule alone. Additionally, these effects of the GLP-1::estrogen conjugates were independent of adverse gynecological and oncogenic outcomes. This strategy appears to uniquely combine potency with specificity; however, the molecular mechanism of these beneficial effects remains to be elucidated. Importantly, these co-agonist data suggest that the maximally achievable and sustainable body-weight loss of 10%, observed with currently available pharmaceutics, may not reflect an insurmountable physiological barrier (Day et al., 2009). Hence, co-agonistic pharmacotherapy as well as varied leptin sensitizers and new classes of chemical compounds hold promise as an efficacious approach in the management of obesity.

Finally, it must be noted that novel centrally acting molecules regulating metabolism are regularly being discovered. Among other examples, FGF21 was recently described as a potent anti-obesity factor predominately affecting energy expenditure (Bookout et al., 2013; Owen et al., 2014; Sarruf et al., 2010) and, in humans, exhibiting a marked lipid-lowering effect (Gaich et al., 2013). Based on genetic studies in rodents, the anti-obesity actions of FGF21 have been attributed to its direct action on the nervous system (Bookout et al., 2013; Owen et al., 2014; Sarruf et al., 2010). However, concurrent data indicate that the adipose tissue, rather than the nervous system, is required for FGF21 anti-obesity actions (Adams and Kharitonenkov, 2012). The regulation of glucose metabolism by FGF21 has also been attributed to direct actions on the liver (Adams and Kharitonenkov, 2012). Further work should paint a fuller picture of FGF21's activities, and overall, the field is hopeful that existing and/or novel ligands currently under development will be efficacious in the treatment of obesity.

Device-Assisted Neuromodulatory Techniques

Device-assisted neuromodulation refers to delivery of an electrical current to either a specific nerve or a particular brain region in order to influence brain activity and autonomic outflow. Devices include but are not limited to stimulators of the spinal cord, vagus nerve, and sacral nerve; deep brain stimulators; and gastric electric stimulators. Although many of these devices have not been approved by the FDA for the treatment of metabolic disorders, accumulating evidence suggests that modulation of targeted brain sites or peripheral nerve activity by device-assisted means is a valuable tool in the treatment of many chronic diseases (Famm et al., 2013).

The idea of being able to target a particular brain site is attractive, considering the unwanted effects of many pharmacological

compounds. Furthermore, peripheral nerves are increasingly recognized to play a critical role in metabolic functions (Bartness et al., 2014; Powley et al., 2005). The vagus nerve is a mixed (sensory and motor) nerve that innervates most of the thoracic and the abdominal viscera, including the entire gastrointestinal tract, pancreas, and liver (Berthoud and Neuhuber, 2000). It is well established that vagal sensory neurons convey a wide variety of signals originating from the gastrointestinal tract, including mechanical stretch, changing levels of nutrients, lipids, immune signals, and gut peptides (de Lartigue et al., 2011). Experimental observations also indicate the reduced ability of vagal afferents to respond to dietary and endogenous metabolic signals in animals fed on high-fat diets (Kentish et al., 2012). These observations strongly support the idea that the vagus nerve serves as a critical link between the gut and the brain and that this link is impaired in obesity. Based on the aforementioned literature linking vagal afferents to post-prandial functions and the regulation of feeding, stimulation of vagal afferents has been hypothesized to be a promising anti-obesity approach and a potential alternative to bariatric surgery (Powley et al., 2005). Pre-clinical studies in large animals and humans suggest that vagus nerve stimulation (VNS) might show efficacy in reducing food craving and weight gain (Pardo et al., 2007; Val-Laillet et al., 2010). A technique of vagal blocking has also been tested in pre-clinical studies (VBLOC) and recently approved by the FDA as a weight-loss treatment device in obese individuals. Briefly, this technique targets the nerve pathway between the brain and the stomach by stimulating the vagal trunks at high frequency, thus interfering with normal gastric functions and leading to early satiation (Camilleri et al., 2008). However, contradictory results have been obtained as to the benefits of this novel weight-loss approach in obese subjects, with a couple of studies suggesting a significant weight loss and reduced food craving (Camilleri et al., 2008; Shikora et al., 2013), and others finding no significant benefits (Ikramuddin et al., 2014; Sarr et al., 2012).

Central neurostimulatory techniques may also be of interest in the treatment of obesity. In particular, high-frequency deep brain stimulation (hfDBS, Table 1) has been effective at treating the symptoms associated with Parkinson's disease and other disabling neurological disorders by normalizing pathological patterns of neuronal activity (Wichmann and Delong, 2006). This technique involves the chronic implantation by stereotaxic surgery of stimulation electrodes in a targeted brain site. Electric current is delivered to electrodes connected to a pulse generator similar to a pace maker. Stimulation of the hypothalamus in humans is feasible and has been recently used in morbidly obese humans to target the LHA (Whiting et al., 2013). Stimulation of the LHA succeeded in increasing resting metabolic rate, leading to reduced binge-eating scores and/or body weight in all three test subjects. Similar results were obtained in a rat model, supporting the idea that this technique could be considered as an option for the treatment of obesity (Soto-Montenegro et al., 2014).

It should be noted that brain surgery is associated with inherent risks of hemorrhage, infection, and post-surgical complication. Less invasive strategies that target the activity of subpopulations of neurons may pose fewer concerns. In particular, transcranial direct current stimulation (tDCS) is emerging as a promising technique for non-invasive neuromodulation in a variety of clinical conditions (Dayan et al., 2013). This approach allows for the modification of neuronal excitability in regions involved in specific behaviors by delivering a weak current through the scalp (Dayan et al., 2013). Thus, tDCS is suited for cortical targets, specifically lateral and dorsomedial sectors of the prefrontal cortex that contribute to cognitive control. Importantly, this technique has shown promise for acutely reducing food craving (Fregni et al., 2008; Montenegro et al., 2012) and may be suitable for obese individuals (Boggio et al., 2009; Truong et al., 2013). Although the field embraces these novel methodologies, others are still needed in order to facilitate therapeutic opportunities in the treatment of various neurological pathologies, including those contributing to obesity.

Treating Diabetes by Targeting the Brain

Many of the currently available drugs to treat T2DM are peripherally acting compounds (Table 1). This includes molecules acting on glucose co-transporters, incretin (gastrointestinal hormone that stimulates a decrease in blood glucose levels) degradation enzymes, nuclear receptor signaling, and bile acid metabolism. The most widely prescribed anti-diabetic drug in the world is Metformin (Glucophage, Glumetza, Glucophage XR, or Fortamet), a suppressant of hepatic glucose production with few severe side effects (Garber et al., 1997). Likely due to their minimal effect on the brain, these peripherally acting agents marginally affect appetite. Overall, the popularity of each of these drugs in the medical community is largely determined by their benefit-risk profile.

Importantly, Claude Bernard's idea that mechanisms behind diabetes may have an origin in the CNS has been largely validated by modern physiological approaches (Obici and Rossetti, 2003; Pocai et al., 2005; Seeley and Tschöp, 2006). Over the past two decades, it has become apparent that many of the currently available anti-diabetic drugs also act directly in the CNS (Table 1 and Figure 2). For instance, Liraglutide and Exenatide are known to act both in the periphery and at the level of the brain (Mul et al., 2013; Sisley et al., 2014). However, one recent study demonstrates that the glucose-lowering effects of Liraglutide are independent of GLP-1 receptor signaling in the brain (Sisley et al., 2014). Instead, the GLP-1 anti-diabetic actions in the periphery have been well studied, as the naturally occurring pancreatic-derived peptide GLP-1 was initially described as an incretin-like peptide that controls blood glucose (Drucker, 2001).

Central 5-HT_{2C} receptor agonism is involved in glycemic control (Berglund et al., 2013; Nonogaki et al., 1998; Xu et al., 2010a), actions that are independent of its effects on food intake and body weight. In particular, deletion of the 5-HT_{2C}R in in mice deficient for the peptide leptin leads to synergistic impairment of glucose balance, independent of additional obesity compared to ob/ob mice (Wade et al., 2008). Furthermore, 5-HT_{2C}R signaling in the hypothalamus has also been linked to glucose metabolism (Xu et al., 2010a). It is therefore conceivable that the reported beneficial effect of Lorcaserin on T2DM (O'Neil et al., 2012) may be linked to its central actions in neurons regulating glycemia.

It is now clear that the anti-diabetic and anti-obesity actions of Mc4 receptors are independent and mediated by distinct neural

substrates. For example, humans deficient for Mc4 receptors are hyperinsulinemic, more so than would be expected from their degree of obesity alone (Farooqi and O'Rahilly, 2005). Recent genetic studies in the mouse have elegantly demonstrated that restoration of Mc4 receptors in brainstem cholinergic neurons normalizes hyperinsulinemia in an Mc4 receptor-deficient background (Myers and Olson, 2012). Conversely, the selective deletion of Mc4 receptors in the cholinergic neurons alone is sufficient to raise insulin levels (Sohn et al., 2013). However, the deletion of Mc4 receptors in both sympathetic and parasympathetic neurons is required in order to lead to hyperglycemia and insulin resistance (Berglund et al., 2014). Hence, Mc4 receptor agonism in the nervous system would appear as a promising anti-diabetic approach if these beneficial effects can be isolated from the aforementioned adverse side effects.

New experimental data indicate that targeting the brain is highly relevant to the treatment of type I diabetes (T1D). Leptin has recently been shown to normalize glucose levels in rodents with T1D, an effect that might be dependent upon leptin receptor signaling in Pomc neurons and the regulation of the hypothalamic-pituitary-adrenal (HPA) axis (Fujikawa et al., 2013; Perry et al., 2014). Surprisingly, leptin appears sufficient to prevent death and restore normoglycemia, even in the absence of insulin therapy (Fujikawa et al., 2013). Leptin has also been shown to be useful for the treatment of other metabolic disorders characterized by hypoleptinemia, which can be the result of congenital or acquired lypodystrophies involving selective loss of fat and, in some cases, severe insulin resistance, dyslipidaemia, hepatic steatosis, and diabetes (Javor et al., 2005; Oral et al., 2002; Petersen et al., 2002). The FDA has approved MYALEPT Metreleptin (trade name: MYALEPT), a synthetically derived analog of leptin, for the treatment of lipodystrophy-related metabolic disorders. Leptin monotherapy in mice (Gavrilova et al., 2000; Shimomura et al., 1999) and in patients suffering from generalized lipodystrophy results in improvements of several metabolic parameters, including lowering of triglyceride and glucose levels as well as hepatic steatosis (Javor et al., 2005; Oral et al., 2002; Petersen et al., 2002). Moreover, after just a few months of leptin therapy, many lipodystrophic patients no longer require pharmacotherapy (e.g., insulin) to regulate glucose levels.

As previously outlined, amylin is an endogenous peptide directly acting on the nervous system and able to potentiate leptin sensitivity (Ravussin et al., 2009). An amylin analog (pramlintide) is approved for T1D and T2DM in combination with insulin under the trade name Symilin (Herrmann et al., 2014). Whereas the mechanism of action of pramlintide on glucose regulation is not well documented, the involvement of the brain in its anti-diabetic actions cannot be ruled out.

At first glance, given the efficacy and large choice of currently available anti-diabetic drugs acting in the periphery, the necessity for developing centrally acting anti-diabetic compounds is not immediately clear. However, it is also evident that centrally acting agents can effectively alleviate diabetes. This may become useful when peripherally acting agents are not well tolerated in certain T2DM patients and also in subjects with poorly controlled T1D. Furthermore, centrally acting agents commonly show a broader spectrum of actions on varied metabolic functions. In particular, the ability of many central agents to simultaneously affect energy expenditure, appetite, cardiovascular function, and glucose metabolism makes them particularly relevant for the treatment of the metabolic syndrome. In summary, the brain should be (re)-considered as a prime target in designing therapeutics for diabetes, especially in individuals with multiple comorbidities.

Is the Brain Involved in the Metabolic Outcomes of Bariatric Surgery?

A number of different approaches to bariatric surgery, also known as gastric bypass, have been described (for review, see Lutz and Bueter, 2014; Stefater et al., 2012). Roux-en-Y gastric bypass is the most efficacious and frequently performed weight-loss surgery (Buchwald, 2014; Lo Menzo et al., 2014; Schauer et al., 2003). However, the use of sleeve gastrectomy has been gaining in popularity in recent years since the approval of laparoscopic sleeve gastrectomy in 2005. Patients eligible for either sleeve gastrectomy or Roux-en-Y gastric bypass can reasonably expect a near normalization of their body mass index and reversal of most co-morbidities within 2 years. Despite their remarkable efficacy, these surgeries remain costly procedures associated with many complications. Perhaps weight-loss surgeries could be simplified and their complications avoided if the biological mechanisms underlying their effects were understood. Thus, many investigators acknowledge that identifying the mechanisms underlying the beneficial effects of gastric bypass is an important challenge. There is now ample evidence that neither the mere physical restriction of the stomach nor nutrient malabsorption is sufficient to explain weight loss and glucose-metabolism improvement after bariatric surgery (Lutz and Bueter, 2014; Stefater et al., 2012). At the physiological level, a combination of modified parameters, including reduced appetite, modified food preference, and augmented energy expenditure, may collectively mediate weight loss after bariatric surgery. At the cellular level, numerous non-exclusive factors have been implicated in the benefits of bariatric surgery, including, but not limited to, elevated levels of gut peptides and bile acids secretion, enhanced intestinal growth, and changes in the microbiome composition (Lutz and Bueter, 2014). At the molecular level, farsenoid-X receptor (FXR), a nuclear receptor for bile acids, has been recently demonstrated to play a key role in mediating weigh loss after Roux-en-Y gastric bypass (Ryan et al., 2014). Interestingly, a recent study further demonstrated that intestinal FXR agonism is sufficient to attenuate diet-induced obesity and insulin resistance in the mouse (Fang et al., 2015).

The role played by the brain in mediating the benefits of bariatric surgery remains open to discussion. At the phenomenological level, the absence of compensatory hunger after bariatric surgery in the face of considerable weight loss suggests major adaptations in hunger pathways and the neural circuits involved in the regulation of body-weight set-point. Whereas the nature of these changes remains unknown, numerous experimental and clinical observations indicate that profound adaptations occur in the gut-to-brain axis after bariatric surgery: (1) Gastric bypass surgery significantly improves post-prandial functions (Borg et al., 2006; Thirlby et al., 2006), suggesting a modified functioning of the gut-brain axis. (2) Augmented c-Fos expression in the brainstem in response to gavage administration of lipid

emulsion (trade name: Intralipid) further supports the view that vagal sensory neurons may behave differently after sleeve gastrectomy (Chambers et al., 2012). (3) Following Roux-en-Y gastric bypass and sleeve gastrectomy, animals eat more frequent meals of smaller size (Stefater et al., 2010; Zheng et al., 2009); a similar phenomenon has been described in humans after Roux-en-Y gastric bypass (Laurenius et al., 2012). (4) Roux-en-Y gastric bypass in rats reduces the excitability of vagal efferents (Browning et al., 2013). Furthermore, Mc4 receptor signaling in mouse vagal efferents is required for diabetes reversal after Roux-en-Y gastric bypass (Zechner et al., 2013). (5) Destroying sensory afferents using capsaicin largely prevents the anti-diabetic actions of entero-gastro anastomosis in rats (Troy et al., 2008). (6) Indirect evidence also suggests that the activity of high-order brain sites may be modified after bariatric surgery. For instance, altered food preference (Shin et al., 2013; Wilson-Pérez et al., 2013) and increase in the risk of alcoholism after gastric bypass in humans (Ostlund et al., 2013; Suzuki et al., 2012) strongly suggest modified reward functions. Brain-imaging studies have also revealed altered dopamine receptor 2 availability after Roux-en-Y gastric bypass (Steele et al., 2010). (7) One study reported a reduced hypothalamic activity in response to food cues after gastric banding (Bruce et al., 2012). According to another study (Stefater et al., 2010), sleeve gastrectomy in the rat does not impact the expression levels of hypothalamic neuropeptides. Further research is needed to determine to what extent the vagal and brain changes discussed above may, at least in part, be causally implicated in the benefits of bariatric surgery.

Looking ahead to Unconventional Brain Therapies in Metabolism

In 1974, the stereotaxic destruction of the lateral hypothalamus was attempted in obese humans. It had been firmly anticipated that this procedure would lead to reduced appetite and weight loss, based on the previously described anorectic phenotype after lateral hypothalamus lesions in the rat. Unfortunately, the obese individuals enrolled in this trial did not respond to the procedure. Important ethical and technical constraints have limited progress in this area, and to the best of our knowledge, similar invasive neurosurgical interventions have not been attempted since. Nonetheless, the concept of altering the integrity of select brain circuits to cure obesity still garners support in the scientific community, and at least two major technical avenues for therapeutic intervention are currently being explored.

One involves cell transplantation technologies. For instance, the transplant of leptin-sensitive neurons into the hypothalamus of leptin receptor-deficient mice resulted in a diminished obesity phenotype (Czupryn et al., 2011). The other involves chemo- and optogenetics in which experimenters selectively silence or activate subtypes of neurons and their connections (Lichtman et al., 2008; Deisseroth, 2012; Urban and Roth, 2015). Unfortunately, there is no currently available experimental evidence to suggest that sustained weight loss and/or T2DM reversal could be achieved using this kind of approach, and it remains unclear whether opto- and chemogenetic techniques in their current iterations will be a viable therapeutic in the treatment of various diseases in humans. However, the following observations offer clues that the application of these technologies to the human brain may be feasible in the near future: (1) Virally mediated gene delivery has been attempted on many occasions in the non-human primate brain and at least once in the human brain. (2) Clozapine-N-oxide, an exogenous ligand commonly used in chemogenetic experiments, has been safely administered to humans. (3) The applicability of optogenetics to the non-human primate brain has been validated. Certainly, for both transplantation and acute manipulation of selectively labeled neurons, ethical and safety concerns will be an important consideration for clinical application.

Conclusions

In order for currently available drug therapies and weight-loss surgeries to be maximally effective in severely obese subjects, a careful nutritional management and monitoring of dietary habits is required. A major goal of current obesity research is to identify pharmacological compounds that would mimic the effect of bariatric surgery without the inherent complexity and potential risks of the surgery. New drugs are continuously in development, but to date, none have reached the efficacy and spectrum of action of bariatric surgery. It is admittedly disappointing that the tremendous progress in our understanding of the neural pathways regulating metabolism has not yet resulted in more clinically relevant progress and clearly not a cure for obesity or T2DM. Nonetheless, the burgeoning field of combinatorial pharmacology, device-assisted neuromodulation medicine, and chemo- and optogenetics holds promise for more effective and safer anti-obesity and anti-diabetic therapies. In parallel, it is necessary to treat the symptoms and complications of obesity with appropriate pharmacological and surgical means. The field should also address the biology underlying human preferences for hypercaloric food, especially given the constant or growing availability of these foods throughout the world. Undoubtedly, an increased understanding of the brain pathways regulating energy balance and glucose homeostasis will provide insights that will facilitate the development of multi-faceted approaches to combat both obesity and diabetes.

ACKNOWLEDGMENTS

This work was supported by grants to K.W.W. (NIH R01 DK100699), L.G. (ANMS Research Grant 2014), and J.K.E. (R01DK53301, R01DK088423, and RL1DK081185).

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Cell

Leading Edge
Review

Immune Regulation of Metabolic Homeostasis in Health and Disease

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http://dx.doi.org/10.1016/j.cell.2015.02.022

Obesity is an increasingly prevalent disease worldwide. While genetic and environmental factors are known to regulate the development of obesity and associated metabolic diseases, emerging studies indicate that innate and adaptive immune cell responses in adipose tissue have critical roles in the regulation of metabolic homeostasis. In the lean state, type 2 cytokine-associated immune cell responses predominate in white adipose tissue and protect against weight gain and insulin resistance through direct effects on adipocytes and elicitation of beige adipose. In obesity, these metabolically beneficial immune pathways become dysregulated, and adipocytes and other factors initiate metabolically deleterious type 1 inflammation that impairs glucose metabolism. This review discusses our current understanding of the functions of different types of adipose tissue and how immune cells regulate adipocyte function and metabolic homeostasis in the context of health and disease and highlights. We also highlight the potential of targeting immuno-metabolic pathways as a therapeutic strategy to treat obesity and associated diseases.

Introduction

Obesity is in an increasingly prevalent metabolic disease characterized by excess accumulation of adipose tissue. Obesity increases the risk of developing a wide variety of diseases including but not limited to type 2 diabetes, cardiovascular diseases, and multiple forms of cancer and has been strongly associated with increased mortality (Prospective Studies Collaboration, 2009; Flegal et al., 2013; Oliveros and Villamor, 2008; Pi-Sunyer, 1999; Pontiroli and Morabito, 2011; Reilly and Kelly, 2011; Rodriguez et al., 2001). In the past few decades, the prevalence of obesity has risen dramatically in both industrialized and less industrialized nations across all continents (Kelly et al., 2008; Ng et al., 2014), and this has been associated with high healthcare expenditures (Withrow and Alter, 2011). For example, in the U.S. in 2009–2010, obesity afflicted 36% of adults (Flegal et al., 2012; Ogden et al., 2012, 2014) and accounted for approximately $190 billion in annual healthcare costs, representing nearly 20% of total national healthcare expenditures that year (Cawley and Meyerhoefer, 2012; Finkelstein et al., 2009). More recent statistics indicate that 35% of adults in the U.S. were obese in 2011–2012 but was as high as 48% in some segments of the population (Ogden et al., 2014). Therefore obesity is a critical problem with major health and economic consequences. Increasing our understanding of the pathways involved in the development of obesity will be critical for the development of new intervention strategies to prevent or treat this disease and its associated co-morbidities.

As in many chronic inflammatory diseases, genetic and environmental factors are important for the development of obesity and associated diseases (Bouchard, 2008; Brestoff and Artis, 2013; McCarthy, 2010; Walley et al., 2009). In addition, emerging studies have implicated various cell types of the immune system as critical regulators of metabolic homeostasis (Jin et al., 2013; Lumeng and Saltiel, 2011; Odegaard and Chawla, 2011, 2013b; Osborn and Olefsky, 2012). Seminal studies connecting the immune system to metabolic dysfunction in obesity indicated that tumor necrosis factor-α (TNF-α) production was upregulated in obese mice and that neutralization of TNF-α improved glucose uptake in murine obesity (Hotamisligil et al., 1993). Subsequent studies revealed that mice lacking TNF-α were protected from high-fat-diet-induced insulin resistance (Uysal et al., 1997). Increased TNF-α production was also observed in human obesity, and weight loss in humans was associated with decreased TNF-α levels (Hotamisligil et al., 1995; Kern et al., 1995). Later, it was discovered that pro-inflammatory macrophages accumulate in adipose of obese mice and that these cells were dominant sources of TNF-α to promote insulin resistance (Weisberg et al., 2003; Xu et al., 2003). Collectively, these studies revealed that obesity is associated with chronic low-grade inflammation and suggested that inflammatory responses can have detrimental metabolic consequences. It is now appreciated that in obesity chronic low-grade inflammation occurs in many organs including but not limited to white adipose tissue (WAT), brown adipose tissue (BAT), pancreas, liver, brain, muscle, and intestine (Cildir et al., 2013). Of these, WAT is the most studied organ in terms of immune-metabolic interactions in obesity.

In WAT, which coordinates metabolism at distant tissues such as the brain, liver, pancreas, and muscle, there is a diverse set of immune cells at steady state (Exley et al., 2014; Ibrahim, 2010; McNelis and Olefsky, 2014; Mraz and Haluzik, 2014). This

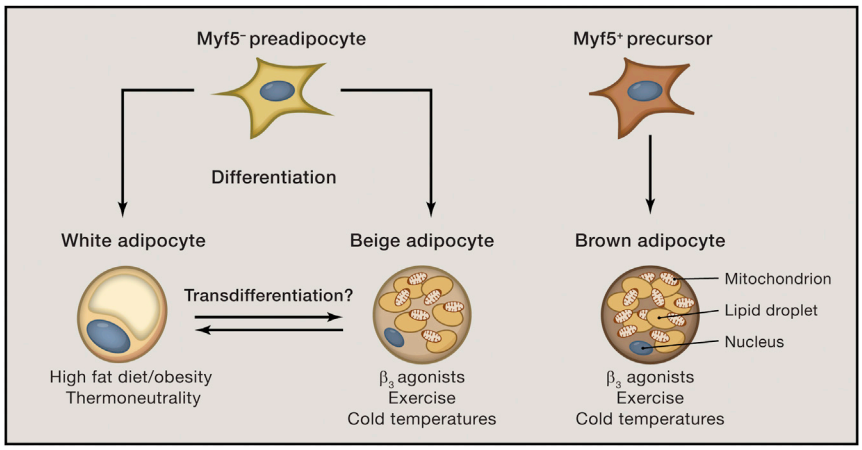

Figure 1. White, Beige, and Brown Adipocytes Are Developmentally and Functionally Distinct Cell Populations

White and beige adipocytes arise from a Myf5⁻ precursor cell population that is bipotent. These pre-adipocytes give rise to white or beige adipocytes depending on the stimulus and physiologic setting. White adipocytes are promoted by high-fat-diet feeding or obesity and by thermoneutrality (30°C in mice). Beige adipocytes are elicited by β_3 adrenergic receptor agonists such as norepinephrine or epinephrine, and are recruited within WAT in the settings of chronic exercise or exposure to cold temperatures. Although white and beige adipocytes emerge from pre-adipocytes via cell differentiation, mature white and beige adipocytes might undergo a process called transdifferentiation, in which one cell type acquires phenotypic characteristics of the other. There is uncertainty about whether transdifferentiation occurs. In contrast, brown adipocytes arise from a Myf5⁺ precursor cell population and are present in discrete brown adipose tissue depots. Despite being developmentally distinct cell populations, beige and brown adipocytes are activated by similar physiologic stimuli, including exercise- and cold temperature-induced hormones and metabolites.

network of immune cells appears to be poised to recognize, integrate, and respond to environmental signals including bacterial products, endogenous lipid species and hormones in order to regulate metabolism (Odegaard and Chawla, 2013a). Changes in immune cell composition and function in WAT have been closely associated with obesity and the regulation of metabolic homeostasis, and disruption of this network of immune cells can have either detrimental or beneficial effects on mammalian health (Exley et al., 2014; Lumeng and Saltiel, 2011; Mraz and Haluzik, 2014; Odegaard and Chawla, 2013b; Osborn and Olefsky, 2012). In addition, recent work has demonstrated that immune-system-associated transcription factors including but not limited to nuclear factor-κ B (NK-κB), c-Jun kinase (JNK), and interferon regulatory factor 4 (IRF4) are key regulators of metabolic homeostasis (Lumeng and Saltiel, 2011; Osborn and Olefsky, 2012). Conversely, metabolite-sensing receptors such as peroxisome proliferator-activated receptor (PPAR)-γ, farnesoid X receptor (FXR), liver X receptor (LXR), G protein coupled receptor 120 (GPR120), and carbohydrate-responsive element binding protein (ChREBP) among others have been shown to regulate immune responses (Brestoff and Artis, 2013; Glass and Saijo, 2010; Hotamisligil and Erbay, 2008; Shoelson et al., 2007; Zelcer and Tontonoz, 2006). Therefore dissecting the complex interactions between immune and metabolic systems will provide important insights into the biology underlying obesity and have implications for understanding how current and future therapeutics might influence metabolism.

The purpose of this review is to describe our current understanding of how immune cells in adipose tissue regulate metabolism. First, we will summarize recent advances in understanding the roles of white, beige, and brown adipose tissues in the regulation of weight gain. Second, we will describe the immune cell composition of adipose tissue at steady state and discuss how these immune cell pathways interact and contribute to the maintenance of metabolic homeostasis. Third, we will discuss immunologic changes that occur in adipose in the setting of obesity and highlight how these changes contribute to metabolic dysfunction. Finally, we will discuss potential therapeutic implications of targeting the immune system to treat obesity and its associated diseases.

Roles of Adipose Tissues in the Regulation of Obesity

Mammals possess multiple types of adipose tissues including white, brown, and beige adipose. These tissues are found in distinct anatomic locations and are comprised of different adipocyte cell types—white, beige, and/or brown—that have unique developmental and functional properties that are critical for host metabolism (Figure 1) (Bartelt and Heeren, 2014; Cannon and Nedergaard, 2004; Harms and Seale, 2013; Ibrahim, 2010; Peirce et al., 2014; Pfeifer and Hoffmann, 2015; Wu et al., 2013). This section describes white, brown, and beige adipocyte cell types and summarizes their roles in regulating weight gain.

White Adipocytes

WAT is distributed throughout the mammalian body in subcutaneous depots and in association with organs, where it has important roles in insulation and physical protection of the viscera, and is comprised predominantly of white adipocytes (Peirce et al., 2014; Pfeifer and Hoffmann, 2015). These specialized cell types arise from a Myf5⁻ pre-adipocyte lineage and store large amounts of triglycerides in a single large lipid droplet (Pfeifer and Hoffmann, 2015; Sanchez-Gurmaches and Guertin, 2014). In addition to their ability to store triglycerides, white adipocytes respond to hormonal signals to induce lipolysis and release free fatty acids (FFA) into the circulation for oxidation or storage by other cell types (Arner et al., 2011; Bartness et al., 2010). Therefore white adipocytes are critical for regulating both fat storage and release. Beyond this function, white adipocytes produce various adipocyte-specific hormones (also known as adipokines) including but not limited to leptin, resistin, retinol binding protein 4 (RBP4), fibroblast grown factor 21 (FGF21), and adiponectin that regulate metabolic homeostasis by acting on distant organs such as the brain, kidney, liver, pancreas, and skeletal muscle (Allison and Myers, 2014; Itoh, 2014; Kadowaki et al., 2006; Kershaw and Flier, 2004; Kotnik et al., 2011; Lazar, 2007; Ouchi et al., 2011).

In the context of obesity, mature white adipocytes undergo proliferation and become hypertrophic through accumulation of triglycerides to increase the number and size of the adipocyte pool in WAT (Foster and Bartness, 2006; Hausman et al., 2001; Kubota et al., 1999; Spalding et al., 2008). As WAT expands, adipocytes increase production of leptin to suppress food intake and therefore limit the rate of triglyceride accumulation and adipocyte expansion (Allison and Myers, 2014). In obesity, this food intake-suppressive effect of upregulated leptin production is blunted by the development of resistance to leptin action (Park and Ahima, 2015). Leptin also has pro-inflammatory effects by eliciting type 1 effector cytokine production by immune cells (Attie and Scherer, 2009; Gregor and Hotamisligil, 2011; Mraz and Haluzik, 2014; Ouchi et al., 2011). In addition to leptin, the adipokines RBP4 and resistin are increased in obesity and promote insulin resistance in mice (Kotnik et al., 2011; Lazar, 2007; Moraes-Vieira et al., 2014; Norseen et al., 2012; Steppan et al., 2001; Yang et al., 2005). Upregulation of RBP4 expression has been linked to inappropriate sensing of low glucose levels by adipocytes (Yang et al., 2005), whereas resistin production is increased due to elevated levels of insulin and TNF-α among other factors (Lazar, 2007; Song et al., 2002). Both of these factors appear to promote insulin resistance in mice, at least in part, through elicitation of type 1 immune responses in WAT (Jung and Choi, 2014; Ouchi et al., 2011). Thus, leptin, resistin, and RBP4 are pro-inflammatory factors that are increased in obesity and that stimulate type 1 immunity in WAT.

In contrast, adiponectin has anti-inflammatory and insulin-sensitizing effects (Gregor and Hotamisligil, 2011; Kadowaki et al., 2006; Lumeng and Saltiel, 2011). Adiponectin production by white adipocytes is decreased in the context of obesity (Ouchi et al., 2011), and loss of this anti-inflammatory signal may be one factor that contributes to the development of inflammation in WAT in obesity. Other adipokines are also emerging as potential anti-inflammatory factors. In particular, recent work suggests that FGF21 might have immunosuppressive effects through inhibition of the transcription factor nuclear factor-κB (NF-κB) (Yu et al., 2015), a master regulator of inflammatory gene expression. Additional research is needed to determine whether FGF21 regulates inflammation in obesity and whether the immune system regulates expression of FGF21. Other adipokines such as fatty acid binding protein 4 (also known as adipocyte protein 2) and neuregulin 4 (NRG4) that have important roles in regulating metabolism have also been associated with inflammatory markers (Cao et al., 2013; Terra et al., 2011; Wang et al., 2014). Future research will be needed to determine whether these adipokines are direct pro- or anti-inflammatory mediators in WAT. Therefore, white adipocytes appear to link metabolic status of mammals to immune responses in WAT.

Brown Adipocytes

Although brown adipocytes can also produce adipokines, these cells differ from white adipocytes developmentally and functionally and in their anatomic distribution. In mice, brown adipocytes arise from a Myf5$^+$ lineage and are developmentally more closely related to skeletal muscle cells than to white adipocytes (Kajimura et al., 2009; Seale et al., 2008). The primary function of brown adipocytes in both mice and humans is to convert chemical energy into heat via uncoupling protein 1 (UCP1) (Cannon and Nedergaard, 2004). UCP1 is a long chain fatty acid/proton symporter that dissipates the mitochondrial electrochemical gradient, thereby uncoupling energy substrate oxidation from ATP synthesis and resulting in the generation of heat (Fedorenko et al., 2012; Matthias et al., 2000). This process is critical for maintaining core body temperature and in the regulation of weight gain by increasing whole-body energy expenditure. In the latter case, for example, mice that lack UCP1 develop obesity when housed at thermoneutrality (30°C) as compared to UCP1-sufficient controls (Feldmann et al., 2009). Although global deletion of UCP1 impairs thermogenesis in both brown and beige adipocytes (see below), brown adipose tissue (BAT) is believed to be an important regulator of metabolic rate and obesity susceptibility in mice.

Brown adipocytes are found in discrete BAT depots. In mice, the largest BAT depot is present in the interscapular space, and smaller depots are present in the axilla and along the cervical column (Bartelt and Heeren, 2014). In humans, brown adipocytes are abundant in newborns including in the interscapular space (Hondares et al., 2014), whereas in lean adult humans brown adipocytes are reported to be present in the supraclavicular space of the neck and adjacent to the cervical column and heart (van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009). Subsequent studies have determined that supraclavicular neck BAT in healthy adults exhibits a gene expression profile and has morphologic characteristics consistent with those of beige adipocytes (Lee et al., 2014a; Sharp et al., 2012; Wu et al., 2012).

Beige Adipocytes

Like brown adipocytes, beige adipocytes (also known as brown-like or brite adipocytes) express high levels of UCP1 and store triglyceride in one or more lipid droplets, giving rise to a paucilocular or multilocular cell morphology. Although beige and brown adipocytes have similar UCP1-dependent functional characteristics, these cell types differ from each other developmentally and anatomically. Brown adipocytes arise from a Myf5$^+$ precursor and are developmentally related to skeletal muscle cells (Kajimura et al., 2009; Seale et al., 2008), whereas beige adipocytes arise from Myf5$^-$ precursors within WAT and are developmentally related to smooth muscle cells (Long et al., 2014) and/or white adipocytes (Sanchez-Gurmaches and Guertin, 2014). Beige adipocytes may arise from multiple developmental pathways, including differentiation from committed beige pre-adipocytes as well as white-to-beige transdifferentiation in which mature white adipocytes are converted into beige adipocytes (Figure 1) (Barbatelli et al., 2010; Harms and Seale, 2013; Moisan et al., 2015; Sanchez-Gurmaches and Guertin, 2014; Wang et al., 2013b; Wu et al., 2012). While differentiation is a well-accepted developmental pathway for beige adipocytes, white-to-beige transdifferentiation is a controversial topic, and further research is needed to determine whether this process contributes to beige adipogenesis in different physiologic contexts or in response to different stimuli. In mice, beige adipocytes are enriched in subcutaneous WAT compared to visceral fat. However, beige adipocytes are elicited in both types of WAT (Qiu et al., 2014), and future studies will be required to determine whether beige adipocytes in subcutaneous and visceral fat differ from each other developmentally and/or functionally.

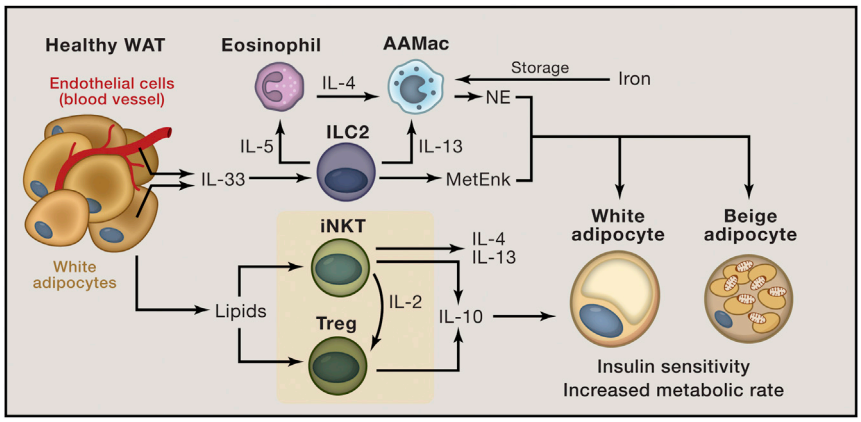

Figure 2. Healthy White Adipose Tissue Is Enriched in Type 2 Cytokine-Associated Immune Cells

In the lean state, adipocytes and endothelial cells in white adipose tissue (WAT) constitutively produce interleukin (IL)-33 that can act on Group 2 innate lymphoid cells (ILC2s) to induce production of IL-5 and IL-13 that sustain eosinophil and alternatively activated macrophage (AAMac) responses, respectively, in WAT. In addition, eosinophils produce IL-4 that is necessary to maintain AAMac responses in WAT. AAMacs have multiple functions to maintain metabolic homeostasis, including storing large amounts of iron, leading to sequestration of this pro-oxidative metal cation from adipocytes to prevent lipid peroxidation, oxidative damage to proteins and mitochondrial dysfunction. In addition, AAMacs produce norepinephrine (NE) that acts on both white and beige adipocytes via the β_3 adrenergic receptor to stimulate lipolysis and elicit beige adipocytes that increase metabolic rate. ILC2s can also promote beiging through production of methionine-enkephalin (MetEnk) peptides. In addition, adipocytes present lipid antigen to invariant natural killer T (iNKT) cells to elicit a unique tissue-specific anti-inflammatory phenotype characterized by increased production of IL-4 and IL-13, which may act on AAMacs, and IL-10. Lipids from adipocytes are also believed to promote regulatory T cell (T_{reg}) responses in WAT to induce production of IL-10. iNKT cells are critical sources of IL-2 and are necessary to sustain T_{regs} in WAT. IL-10 production by iNKT cells, T_{regs} and other cell types promote insulin action in white adipocytes to facilitate maintenance of an insulin-sensitive state. Together, these pathways contribute to metabolically healthy WAT.

Beige adipocytes are elicited in response to environmental stimuli including exposure to cold environmental temperatures or in response to exercise, and these cells are activated or induced to differentiate in response to a range of small molecules and hormones. Factors that stimulate beige adipocyte development or activation include norepinephrine (NE) (Qiu et al., 2014; Rao et al., 2014), adenosine (Gnad et al., 2014), lactate (Carrière et al., 2014), β-aminoisobutyric acid (BAIBA) (Roberts et al., 2014), prostaglandin E_2 (PGE_2) (Madsen et al., 2010), parathyroid hormone-related protein (PTHrp) (Fisher et al., 2012), bone morphogenetic protein 4 (BMP4) (Qian et al., 2013), BMP7 (Schulz et al., 2011), fibroblast growth factor 21 (FGF21) (Fisher et al., 2012), irisin (Wu et al., 2012), and meteorin-like (Rao et al., 2014). Although many factors are known to promote beige adipocyte differentiation or function, it remains unknown whether these factors act on different subsets of beige adipocytes or whether they elicit developmentally or functionally distinct subsets of beige adipocytes. In addition, it is unclear to what extent these factors coordinately regulate beige adipocyte function.

Nonetheless, recent studies indicate that selective deletion of beige but not brown adipocytes results in impaired glucose homeostasis and increased susceptibility to weight gain in response to high fat diet (HFD) feeding (Cohen et al., 2014). Conversely, mice with increased beige adipocytes exhibit increased metabolic rate and decreased obesity (Wang et al., 2013a). Consistent with this finding, 129Sv mice, which are resistant to diet-induced obesity exhibit increased beiging of WAT compared to C57BL/6 mice, which are susceptible to diet-induced obesity (Shabalina et al., 2013). Inhibition of beige adipocytes by supplementing HFD chow with indomethacin resulted in increased weight gain following HFD feeding in 129Sv mice (Madsen et al., 2010). Collectively, these data indicate that impaired beiging is associated with increased weight gain and suggest that elicitation of beige adipocytes can combat obesity in mice.

Recent studies support the relevance of these findings in humans. In the context of human obesity, the incidence of brown/beige adipose tissue (Sharp et al., 2012) is significantly decreased in obese patients compared to lean patients (Saito et al., 2009). In addition, UCP1 expression was found to be higher in cultured beige adipocytes from lean patients compared to obese patients, suggesting that beige adipocyte differentiation or activation is impaired in human obesity (Carey et al., 2014). Consistent with this, ephedrine-induced activation of brown/beige adipose tissue is impaired in human obesity (Carey et al., 2013). While the role of brown/beige adipocytes in the regulation of energy expenditure in humans is unclear, these studies suggest that obesity is associated with loss of beige fat function and raise the possibility that defective beige fat may have deleterious metabolic effects including increased susceptibility to weight gain.

Immune Cell Composition and Function in Lean White Adipose Tissue

White adipose is a heterogeneous tissue comprised of multiple cell types including a diverse array of immune cells. In the steady state, these immune cells tend to be associated with the type 2 immune axis and include alternatively activated macrophages (AAMacs), eosinophils, Group 2 innate lymphoid cells (ILC2s), invariant natural killer T (iNKT) cells, T helper type 2 (Th2) cells, regulatory T (T_{reg}) cells, and other immune cell populations that communicate with each other and participate in cross-talk with adipocytes. As discussed below, the interface between immune cells and adipocytes contributes to the regulation of lipid storage, glucose utilization, redox balance, and energy expenditure (Figure 2). However, in the context of obesity, type 2 immune cells in WAT become dysregulated and, in some cases, acquire a pro-inflammatory phenotype that exacerbates adipose tissue inflammation with deleterious effects on metabolism. In this section, we discuss emerging concepts in the composition and function of immune cells in WAT at steady state and how these cells contribute to protection from diet-induced obesity.

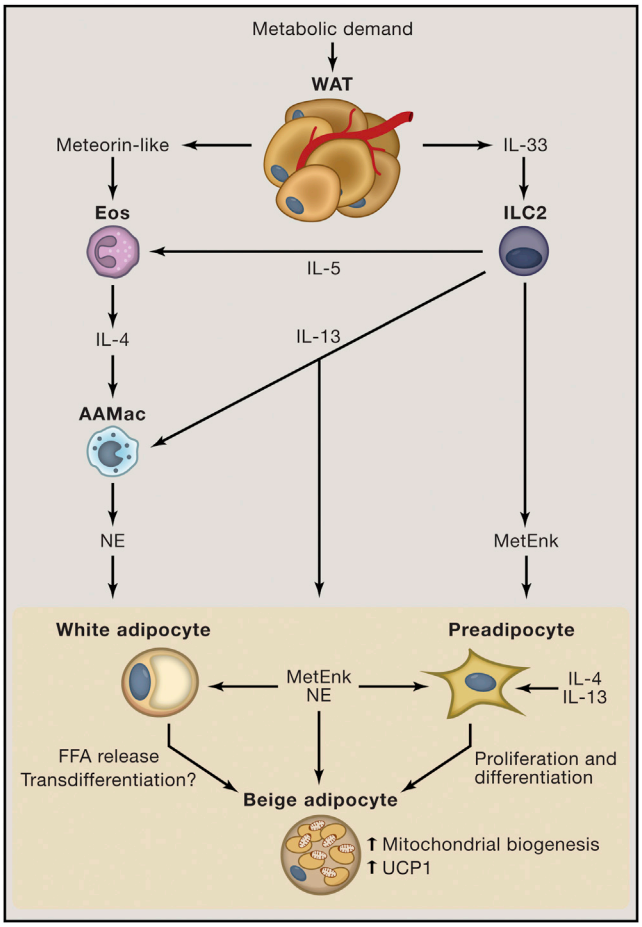

Figure 3. Immunologic Mechanisms That Regulate Beiging

In the context of chronic exposure to cold environmental temperatures or chronic exercise, WAT and muscle produce the adipokine/myokine meteorin-like. This hormone and other factors such as IL-5 promote eosinophil accumulation in WAT. Eosinophils produce IL-4 that sustains alternatively activated macrophages (AAMacs). IL-4 and perhaps IL-13 act on AAMacs to stimulate norepinephrine (NE) production. NE acts to stimulate beiging via differentiation and/or transdifferentiation pathways and to activate existing beige adipocytes, resulting in mitochondrial biogenesis, uncoupling protein 1 (UCP1) upregulation, and UCP1-dependent increases in energy expenditure. In addition, WAT produces the cytokine IL-33 that is critical for maintaining ILC2 responses in WAT. IL-33 stimulates ILC2s to produce IL-5 and IL-13 that sustain the eosinophil/AAMac pathways that can contribute to beiging. In addition, IL-13 can act on pre-adipocytes to promote their proliferation and induce differentiation to beige adipocytes. Further, IL-33 stimulates ILC2s to produce methionine-enkephalin (MetEnk) peptides that can directly promote beiging. Therefore AAMacs and ILC2s both contribute to beiging through production of distinct effector molecules.

The IL-4/Alternatively Activated Macrophage Pathway

AAMacs are IL-4- and IL-13-dependent cells that require signal transducer and activator of transcription 6 (STAT6) to maintain an alternative activation state that is distinct from the classical activation state induced by interferon-γ (IFNγ) (Gordon and Martinez, 2010; Hill et al., 2014; Martinez et al., 2009; Odegaard and Chawla, 2011; Olefsky and Glass, 2010). AAMacs are critical for protective immunity against helminth pathogens and are associated with pathologic allergic inflammation in the lung (Gordon and Martinez, 2010; Martinez et al., 2009). Recent studies have also implicated AAMacs in the regulation of metabolic homeostasis (Hill et al., 2014; Odegaard and Chawla, 2011; Olefsky and Glass, 2010). Early studies linking AAMacs to metabolism demonstrated that macrophage-specific deletion of Peroxisome proliferator-associated receptor (PPAR)-γ results in decreased AAMacs in WAT in association with increased weight gain, elevated fat accumulation, impaired glucose uptake and insulin resistance following HFD feeding (Odegaard et al., 2007). In addition, deletion of PPAR-δ in leukocytes also impaired AAMac responses and was associated with increased adiposity and insulin resistance (Odegaard et al., 2008). These loss-of-function studies are complemented by gain-of-function approaches indicating that administration of exogenous IL-4 to mice to boost AAMac responses protected mice from HFD-induced obesity and insulin resistance (Chang et al., 2012; Ricardo-Gonzalez et al., 2010). Together, these studies identified a critical role for the IL-4/AAMac axis in the regulation of metabolic homeostasis.

Emerging work has revealed that AAMacs employ a diverse functional repertoire to regulate metabolism. For example, a subset of AAMacs in WAT can express genes involved in iron handling and has the capacity to store large amounts of iron in lean mice (Orr et al., 2014). The capacity of these specialized AAMacs to store iron is diminished in the setting of obesity, leading to abnormal accumulation of iron in adipocytes (Orr et al., 2014) that may lead to iron-initiated production of highly toxic lipid aldehydes that damage cellular proteins, impair adipocyte insulin sensitivity, and lead to mitochondrial dysfunction (Curtis et al., 2010; Curtis et al., 2012). Therefore it appears that specialized iron-storing AAMacs have an essential role in regulating iron homeostasis in WAT to maintain optimal glucose metabolism and mitochondrial function at steady state, and dysregulation of these cells in obesity may have deleterious effects on glucose and energy homeostasis. Consistent with this, treatment of obese mice with the iron chelator deferoxamine is associated with improved glucose tolerance, decreased body weight, lower fat mass, and less inflammation in fat compared to vehicle-treated obese controls (Tajima et al., 2012).

In addition, AAMacs from adipose are capable of producing catecholamines such as norepinephrine (NE) that act on adipocytes via the β_3 adrenergic receptor (β_3AR) to stimulate lipolysis, activate mitochondrial biogenesis and upregulate expression of UCP1 (Figures 2 and 3) (Liu et al., 2014; Nguyen et al., 2011; Qiu et al., 2014; Rao et al., 2014). AAMac-derived catecholamine production was described in the setting of brown adipocyte activation (Nguyen et al., 2011). Mice that lack IL-4 and IL-13 (*Il4/13*$^{-/-}$); the shared receptor for these cytokines, IL-4Rα (*Il4ra*$^{-/-}$); or STAT6, a transcription factor downstream of IL-4Rα signaling (*Stat6*$^{-/-}$), had defective AAMac responses (Martinez et al., 2009) and exhibited decreased UCP1 expression in BAT (Nguyen et al., 2011). This defect in UCP1 expression in BAT was associated with impaired maintenance of core body temperature following acute exposure to cold environmental temperatures over a 6 hr period compared to wild-type controls (Nguyen et al., 2011). These findings suggested that IL-4/IL-4Rα-dependent activation of AAMacs stimulated production of NE that was necessary for brown adipocyte activation in the setting of short-term cold exposure. However, subsequent

studies indicated that IL-4/IL-4Rα-dependent AAMacs were dispensable for brown adipocyte activation in the setting of more prolonged cold exposure over 72 hr (Qiu et al., 2014). This suggests that there may be time-dependent factors that influence whether the IL-4/AAMac/NE pathway activates brown adipocytes, a topic that should be investigated further.

The IL-4/AAMac/NE pathway is also critical for activating beige adipocytes in WAT (Figure 3) (Qiu et al., 2014; Rao et al., 2014). Mice lacking IL-4/IL-13, IL-4Rα, or STAT6 failed to undergo optimal beiging of WAT and had decreased metabolic rate compared to wild-type controls after 72 hr of exposure to cold environmental temperatures (Qiu et al., 2014; Rao et al., 2014). This phenotype appears to be mediated by macrophage-intrinsic production of NE, as mice that lack the rate-limiting step of catecholamine synthesis, tyrosine hydroxylase (TH), in LysM-Cre-expressing cells such as macrophages exhibit impaired beige adipose development and exhibit a 10%–15% decrease in whole-body oxygen consumption in the setting of chronic exposure to mild (room temperature) or severe (5°C) cold environmental temperatures (Qiu et al., 2014). This defect in metabolic rate suggests that AAMac-derived catecholamines might also be important for limiting weight gain. Consistent with this, mice that lack Receptor interacting protein 140 (RIP140), a transcriptional co-regulator that regulates metabolism in various tissues and that suppresses mitochondrial uncoupling (Rosell et al., 2011), had increased AAMac responses in WAT and increased beiging that was associated with protection from diet-induced obesity (Liu et al., 2014). In addition, treatment of mice with exogenous IL-4 elicits beiging and increases oxygen consumption levels in an UCP1-dependent manner, effects that are associated with decreased severity of diet-induced obesity (Chang et al., 2012; Qiu et al., 2014; Ricardo-Gonzalez et al., 2010). In addition to its effects on AAMacs, IL-4 can act directly on preadipocytes to promote beige adipocyte development (Lee et al., 2015). Together, these data suggest that IL-4 in WAT is critical for beiging and regulation of metabolic rate through UCP1-dependent thermogenesis.

Sources of IL-4 in WAT: Eosinophils and Invariant Natural Killer T Cells

The appreciation of AAMacs as critical regulators of metabolic homeostasis sparked interest in determining the cellular sources of IL-4 in WAT. Using the *4get* mouse, a reporter strain that identifies IL-4-competent cells, eosinophils were identified as an abundant immune cell population in WAT and an important source of IL-4 in this compartment at steady state (Wu et al., 2011). Eosinophils are granulocytes that are developmentally dependent on the cytokine IL-5 and the transcription factor GATA-binding protein 1 (GATA-1), and these cells contribute to type 2 immune responses by producing a variety of effector molecules including IL-4 (Rosenberg et al., 2013; Rothenberg and Hogan, 2006). Eosinophils are decreased in WAT of HFD-fed and genetically obese *ob/ob* mice (Wu et al., 2011). *ΔDblGata1* mutants or IL-5-deficient mice, both of which lack eosinophils (Rosenberg et al., 2013; Rothenberg and Hogan, 2006), develop more severe obesity and insulin resistance than wild-type controls in association with dysregulated AAMac responses (Wu et al., 2011). Further, mice with elevated numbers of eosinophils in WAT due to constitutive overexpression of IL-5 or infection with helminth pathogens were protected from diet-induced obesity and insulin resistance (Wu et al., 2011; Yang et al., 2013). Eosinophils are also recruited to WAT in the setting of chronic exposure to cold environmental temperatures, and eosinophil-deficient *ΔDblGata1* mice exhibit defective beiging of WAT (Qiu et al., 2014; Rao et al., 2014). These beneficial metabolic effects of eosinophils appear to be mediated by IL-4-dependent AAMac responses in WAT (Wu et al., 2011; Qiu et al., 2014); however, whether eosinophils have other functions in WAT remains unknown.

In addition to eosinophils, iNKT cells that express lipid antigen-specific T cell receptor variants appear to be an important source of IL-4 in WAT (Hams et al., 2013; Lynch et al., 2012). iNKT cells in WAT produce more IL-4 and IL-10 and less IFN-γ than do iNKT cells in the liver and spleen (Lynch et al., 2012), suggesting that iNKT cells in WAT are in an "alternative activation" state. Consistent with this, recent work has shown that mammals produce endogenous lipid antigens such as α-galactosylceramides (αGalCer) that promote production of type 2 cytokines by iNKT cells (Kain et al., 2014; Lynch et al., 2012), and that adipocytes can present lipid antigens to iNKT cells via CD1d (Huh et al., 2013; Rakhshandehroo et al., 2014). iNKT cells are decreased in murine and human obesity, and surgical or dietary treatment of obesity restores iNKT cells in WAT (Lynch et al., 2012). Genetic studies in mice provide further support for the hypothesis that iNKT cells play a critical role in maintenance of body weight and metabolism. Jα18-deficient mice, which lack iNKT cells, exhibit increased body weight and adiposity when fed a HFD compared to wild-type controls, and CD1d-deficient mice, which cannot present lipid antigen to iNKT cells, develop spontaneous obesity on a low-fat diet (Lynch et al., 2012). In addition, mice lacking iNKT cells had more severe glucose intolerance and increased pro-inflammatory macrophages in WAT (Lynch et al., 2012), suggesting that iNKT cells are essential for limiting inflammatory responses in the setting of obesity. Adoptive transfer of iNKT cells induced weight loss and decreased adipocyte size while improving insulin resistance in HFD-fed mice (Hams et al., 2013; Lynch et al., 2012), and treatment of HFD mice with αGalCer recapitulated these effects in an iNKT-dependent manner by increasing IL-4 and IL-10 production by iNKT cells (Lynch et al., 2012). Therefore, eosinophils and iNKT cells may be important sources of IL-4 or other factors that support AAMac function or that directly regulate metabolic homeostasis to limit obesity and insulin resistance.

The IL-33/Group 2 Innate Lymphoid Cell Pathway

ILC2s are recently described immune cells (Moro et al., 2010; Price et al., 2010; Neill et al., 2010) that control eosinophil and AAMac responses (Molofsky et al., 2013; Nussbaum et al., 2013). ILC2s are members of a broader family of ILCs that comprise T-bet-dependent Group 1 ILCs that produce IFN-γ; GATA-3-dependent ILC2s that produce IL4, IL-5, IL-9, IL-13, and amphiregulin; and RORγt-dependent Group 3 ILCs that produce IL-17A and IL-22 (Kim, 2015; Monticelli et al., 2012; Sonnenberg et al., 2013; Spits et al., 2013; Spits and Cupedo, 2012). At barrier surfaces such as the gut, lung, and skin, ILC2s respond to epithelial cell-derived cytokines IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) to initiate type 2

immune responses that protect against helminth infection or that promote pathologic allergic inflammation (Kim, 2015; Monticelli et al., 2012; Sonnenberg et al., 2013; Spits et al., 2013; Spits and Cupedo, 2012). ILC2s were recently shown to be present in murine WAT, where these cells constitutively produce the effector cytokines IL-5 and IL-13 to maintain eosinophil and AAMac responses, respectively, in WAT (Molofsky et al., 2013). Strikingly, eosinophil-deficient mice exhibit decreased energy expenditure and increased obesity compared to eosinophil-sufficient controls (Molofsky et al., 2013; Wu et al., 2011). Recent studies showed that antibody-mediated depletion of ILC2s is associated with increased weight gain and more severe insulin resistance in mice fed a HFD (Hams et al., 2013), and transferring IL-25-elicited ILC2s was sufficient to promote weight loss in diet-induced obese mice (Hams et al., 2013). These data suggest that ILC2s negatively regulate the development of obesity.

Although ILC2s can regulate metabolic homeostasis through their effects on eosinophils and AAMacs, they also have direct effects on metabolism through production of IL-13 and enkephalin peptides (Brestoff et al., 2015; Lee et al., 2015). In the context of exposure to cold environmental temperatures, ILC2-derived IL-13 promotes pre-adipocyte proliferation and differentiation into beige adipocytes in an IL-4Rα-dependent manner (Lee et al., 2015). The ILC2/IL-13/beiging pathway may also be related to young age-associated increases in beiging that may be critical for maintaining core body temperature during mammalian development (Lee et al., 2015). However, whether ILC2-derived IL-13 regulates obesity remains unknown. In this disease context a distinct ILC2-dependent pathway can promote beiging independently of eosinophils or IL-4Rα (Brestoff et al., 2015). Specifically, ILC2s produce methionine-enkephalin (MetEnk) peptides in response to IL-33 stimulation, and MetEnk can act directly on adipocytes from WAT to upregulate *Ucp1* expression levels in vitro and drive the formation of functional beige adipose tissue in vivo (Brestoff et al., 2015). MetEnk was previously shown to stimulate lipolysis in adipocytes (Nencini and Paroli, 1981), a process that is critical for beige adipocyte responses and UCP1 function (Fedorenko et al., 2012; Harms and Seale, 2013; Rosen and Spiegelman, 2014; Wu et al., 2013). Therefore, ILC2s directly contribute to beiging under various physiologic settings by producing IL-13 and MetEnk.

It is possible that ILC2-derived IL-13 and MetEnk, coupled with AAMac-derived NE, cooperatively support optimal beiging to regulate metabolic homeostasis. Consistent with this hypothesis, previous studies have suggested that NE and MetEnk signaling pathways might interact to cooperatively stimulate lipolysis in adipocytes (Nencini and Paroli, 1981). Another possibility is that IL-13, MetEnk and NE elicit distinct populations of beige adipocytes involved in the regulation of metabolic homeostasis in different physiologic contexts or following different environmental stressors. Future studies focused on IL-13, MetEnk, and NE interactions in adipocytes are warranted to better understand the mechanisms by which ILC2s, eosinophils, and AAMacs contribute to the regulation of beiging and metabolic rate.

ILC2-mediated regulation of metabolic processes may be dysregulated in the context of obesity. The cytokine IL-33 is critical for stimulating proliferation and activation of ILC2s (Kim et al., 2014; Molofsky et al., 2013; Monticelli et al., 2011; Moro et al., 2010; Neill et al., 2010; Price et al., 2010) and is expressed at higher levels in WAT of obese mice and humans compared to non-obese controls (Zeyda et al., 2013). However, ILC2s are decreased in murine and human obesity (Brestoff et al., 2015; Molofsky et al., 2013), suggesting that in the obese state ILC2s may be hypo-responsive to IL-33 or have altered cell death, proliferation, or migration. IL-33 was recently shown to be critical for protecting mice from obesity (Brestoff et al., 2015; Miller et al., 2010). Mice lacking IL-33 or the IL-33R exhibit increased obesity and exacerbated impairments in glucose homeostasis, and treatment of obese mice with recombinant IL-33 limited adiposity and improved glucose metabolism in association with enhanced ILC2 and AAMac responses in WAT (Brestoff et al., 2015; Miller et al., 2010). This suggests that targeting the IL-33/ILC2 axis therapeutically could have beneficial metabolic effects that limit obesity and/or associated diseases such as type 2 diabetes.

CD4⁺ T Helper Cells and Regulatory T Cells

In addition to regulating the ILC2/eosinophil/AAMac pathway, IL-33 also acts on adaptive immune cells such as Th2 cells (Molofsky et al., 2013) and T_{regs} (Schiering et al., 2014). Although Th2 cells are relatively rare in WAT (Molofsky et al., 2013) and remain poorly understood in the context of obesity, T_{regs} in WAT have been shown to be unique members of the T_{reg} pool exhibiting elevated expression of PPAR-γ, Foxp3, and IL-10 compared to T_{regs} in other tissues (Cipolletta et al., 2012; Deiuliis et al., 2011; Feuerer et al., 2009). T_{regs} in WAT contribute to the maintenance of insulin sensitivity in WAT by limiting inflammation and producing insulin-sensitizing factors such as IL-10 (Cipolletta et al., 2012; Feuerer et al., 2009; Ilan et al., 2010). For example, IL-10 suppresses monocyte chemotactic protein-1 (MCP-1) expression by adipocytes to limit inflammatory macrophage infiltration of WAT and inhibits the ability of TNF-α to downregulate glucose transporter 4 (GLUT-4) expression and impair insulin action in adipocytes (Lumeng et al., 2007). In obesity, T_{regs} are decreased in both mice and humans (Cipolletta et al., 2012; Feuerer et al., 2009; Wagner et al., 2013), which may be due to aberrant iNKT cell responses in obese WAT (Lynch et al., 2015). iNKT cells in WAT produce IL-2 to sustain T_{regs} and are an additional source of IL-10 (Lynch et al., 2015; Lynch et al., 2012). Interestingly, recent studies indicate that the anti-diabetic class of drugs known as thiazoladinediones (TZD), which are PPAR-γ agonists, ameliorate insulin resistance in obese mice in part via their effects on T_{regs} (Cipolletta et al., 2012). Therefore promoting T_{reg} responses may be a useful strategy to treat or prevent type 2 diabetes.

Immune Cell Responses in White Adipose Tissue in Obesity

In obesity, WAT undergoes metabolic and inflammatory changes. As white adipocytes accumulate triglycerides and become hypertrophic, the vasculature in WAT becomes rarified leading to hypoxia and oxidative stress (Curtis et al., 2010; Pasarica et al., 2009). In addition, increased oxygen consumption by adipocytes undergoing hypertrophy results in relative local hypoxia that triggers hypoxia-inducible factor 1 α (HIF-1α) activation (Lee et al., 2014b). These changes are associated with increased adipocyte cell death and elevated production of

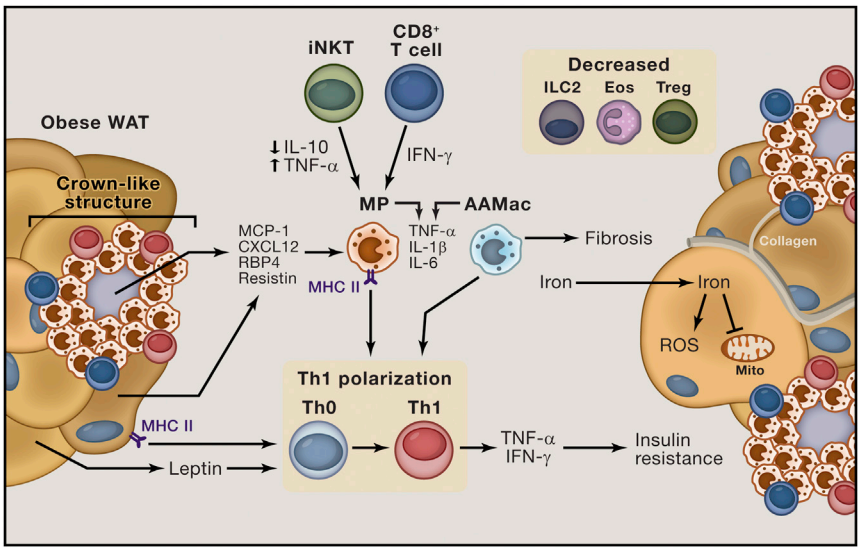

Figure 4. Obese White Adipose Tissue Is Characterized by Type 1 Cytokine-Associated Immune Responses

In obese WAT, adipocyte hypertrophy is associated with hypoxia and adipocyte cell death. Dead and neighboring adipocytes produce pro-inflammatory signals such as Monocyte chemotractant protein 1 (MCP1), C-X-C motif chemokine 12 (CXCL12), retinol binding protein 4 (RBP4) and resistin that are associated with the recruitment of classically activated macrophages (MP) into WAT and MP activation. MP accumulation in WAT is also mediated by pro-inflammatory iNKT cells that exhibit impaired production of IL-10 and upregulated production of tumor necrosis factor-α (TNF-α), and by $CD8^+$ cytotoxic T cells that produce interferon (IFN)-γ. These factors also promote MP activation and upregulate major histocompatibility complex class II (MHC II) on MP and adipocytes. MHC II-mediated antigen presentation by MP and adipocytes stimulates polarization of $CD4^+$ T cells toward a T helper type 1 (Th1) phenotype. Supporting this process are TNF-α, IFN-γ, IL-1β and IL-6 collectively produced by MP cells, dysregulated AAMacs, iNKT cells and $CD8^+$ T cells. In addition, leptin is upregulated in obese WAT, and this factor also promotes Th1 cell polarization. MP cells, $CD8^+$ T cells and Th1 cells collectively interact to form crown-like structures (CLS) to facilitate phagocytosis of dead adipocytes (grey cell in center of CLS). This process further promotes antigen presentation and type 1 immune responses, establishing a vicious cycle. Type 1 cytokines such as TNF-α and IFN-γ act directly on adipocytes to impair insulin action, leading to insulin resistance. Dysregulated AAMacs also produce type 1 cytokines in the setting of obesity to contribute to insulin resistance. AAMacs also lose their capacity to store iron, resulting in redistribution of iron to adipocytes. This results in iron-initiated lipid peroxidation that causes reactive oxygen species (ROS) production, insulin resistance, and mitochondrial dysfunction. In addition, AAMacs in obese WAT promote collagen deposition in WAT and fibrosis, leading ultimately to exacerbated hypoxia and inflammation and potentiation of the type 1 immune response. These processes occur in the setting of decreased abundance of regulatory T cells (T_{reg}), Group 2 innate lymphoid cells (ILC2), and eosinophils (Eos) that promote insulin sensitivity and metabolic homeostasis in WAT in the steady state. Mito, mitochondrion.

adipocyte-derived inflammatory mediators, including the adipokines leptin, resistin and RBP4 (Attie and Scherer, 2009; Greenberg and Obin, 2006; Lazar, 2007; McNelis and Olefsky, 2014; Osborn and Olefsky, 2012; Ouchi et al., 2011). As discussed below, these and other pro-inflammatory factors initiate a type 1 immune response in obese WAT that is characterized by increased accumulation of $CD4^+$ T helper type 1 (Th1) cells, cytotoxic $CD8^+$ T cells, and pro-inflammatory classically activated macrophages (Figure 4). These changes are discussed in this section.

Adipocytes, Macrophages and Type 1 Cytokine-Associated T Cells Participate in a Proinflammatory Positive-Feedback Loop in Obesity

In obesity adipocyte-derived inflammatory mediators such as monocyte chemotactic protein (MCP-1), C-X-C motif chemokine 12 (CXCL12), prostaglandins, leukotrienes, and other factors are increased and promote classically activated monocyte/macrophage activation, proliferation, and infiltration of WAT (Amano et al., 2014; Nomiyama et al., 2007; Oh et al., 2012; Weisberg et al., 2003). These cells engulf dying or dead adipocytes, forming crown-like structures (CLS) that are characterized morphologically as a ring of macrophages and other immune cells surrounding an adipocyte (Murano et al., 2008; Ouchi et al., 2011). The formation of CLS may be an adaptive mechanism to scavenge cellular debris or to limit the release of toxic lipid species when adipocytes undergo cell death via phagocytosis. However, in addition to their phagocytic roles in CLS, classically activated macrophages in WAT of obese mice produce IL-1β, TNF-α, and IL-6 among other factors that potentiate the type 1 inflammatory response (Lumeng et al., 2007; Nguyen et al., 2007; Zeyda et al., 2010). These cytokines act directly on adipocytes and other cell types in distant tissues such as skeletal muscle to inhibit insulin-dependent glucose uptake (Exley et al., 2014; Gregor and Hotamisligil, 2011; Jin et al., 2013; Osborn and Olefsky, 2012). This pro-inflammatory process is therefore associated with the development of insulin resistance by promoting chronic low-grade type 1 inflammation.

The accumulation of macrophages in obese WAT also appears to be regulated by $CD8^+$ T cells (Rausch et al., 2008). These adaptive immune cells are recruited to WAT before infiltration by pro-inflammatory macrophages in the setting of HFD feeding (Nishimura et al., 2009). Deletion of $CD8^+$ T cells decreases macrophage accumulation in WAT of obese mice and ameliorates obesity-associated insulin resistance, and adoptive transfer of $CD8^+$ T cells to mice fed a HFD is sufficient to promote macrophage infiltration of WAT and exacerbate insulin resistance (Nishimura et al., 2009). In the setting of HFD feeding, $CD8^+$ T cells produce IFN-γ that has multiple effects on immune cells in WAT (Revelo et al., 2015). IFN-γ acts on macrophages to upregulate expression of pro-inflammatory effector cytokines and to increase expression of Major histocompatibility complex class II (MHCII) (Schroder et al., 2004). This promotes macrophage antigen presentation to $CD4^+$ T cells and induces Th1 cell polarization and proliferation (Cho et al., 2014; Morris et al., 2013). T-bet-dependent $CD4^+$ Th1 cells produce TNF-α and IFN-γ to further potentiate insulin resistance in WAT (Cho et al., 2014; Morris et al., 2013; Stolarczyk et al., 2013). Thus, the $CD8^+$ T cell/classically activated macrophage pathway in WAT appears to be critical for establishing the pro-inflammatory

environment that influences Th1 cell responses. However, the precise factors that spur this $CD8^+$ T cell-dependent response remain to be determined.

Adipocytes also appear to directly contribute to Th1 cell responses in obesity. With increased triglyceride deposition, adipocytes upregulate their expression of leptin, a hormone that limits food intake, as a compensatory mechanism to guard against overly rapid weight gain (Allison and Myers, 2014). While this is a beneficial response to maintain energy homeostasis, in WAT leptin acts on $CD4^+$ T cells to induce Th1 polarization and IFN-γ expression to drive a pro-inflammatory immune response that limits insulin sensitivity (Deng et al., 2013). In turn, IFN-γ upregulates CIITA and MHCII expression in adipocytes (Deng et al., 2013) and macrophages (Cho et al., 2014; Morris et al., 2013) to promote adipocyte-mediated antigen presentation to Th1 cells. These MHCII-mediated inflammatory changes in obesity are critical drivers of insulin resistance (Cho et al., 2014; Deng et al., 2013). Therefore it appears that adipocytes, macrophages, $CD8^+$ T cells, and $CD4^+$ Th1 cells participate in a pro-inflammatory positive-feedback loop with deleterious consequences for WAT inflammation and glucose metabolism.

Alternatively Activated Macrophages Acquire a Classical Activation State in Obesity and Potentiate Type 1 Inflammation in WAT

As discussed above, AAMacs have critical roles for regulating metabolic homeostasis in WAT at steady state. In obesity, however, AAMacs appear to become dysregulated and acquire a pro-inflammatory classically activated-like phenotype that contributes to the development of type 1 inflammatory responses in WAT. For example, in obesity, AAMacs decrease their expression of IL-10 and Arginase 1 and upregulate expression of TNF-α, IL-6, and IL-1β (Han et al., 2013; Lumeng et al., 2007; Moraes-Vieira et al., 2014). This phenotypic switch in AAMacs appears to be driven, at least in part, by RBP4 that is upregulated in obesity and that impairs glucose homeostasis (Graham et al., 2006; Norseen et al., 2012). AAMacs from transgenic mice that overexpress RBP4 exhibit upregulated expression of antigen presentation machinery, TNF-α and IL-1β, and polarize $CD4^+$ T cells in WAT toward a Th1 phenotype characterized by increased expression of T-bet and IFN-γ (Moraes-Vieira et al., 2014). Interestingly, this effect was not observed in the liver, suggesting a tissue-specific effect of RBP4 on antigen presenting cells and T cell activation (Moraes-Vieira et al., 2014). In addition, transfer of RBP4-activated bone marrow-derived dendritic cells (DCs) (BMDCs) to lean recipient mice was sufficient to promote Th1 cell polarization in WAT and insulin resistance compared to unactivated BMDCs (Moraes-Vieira et al., 2014). This suggests that AAMacs in WAT become dysregulated in the setting of obesity and acquire a pro-inflammatory classical activation state that supports Th1 cell polarization and the development of insulin resistance.

Perspectives and Conclusions

Adipose tissues are diverse in their structure and function and have multiple roles in the regulation of energy balance and weight gain (Figure 1). WAT is essential for triglyceride storage and regulation of glucose homeostasis, and white adipocytes appear to link mammalian metabolic status to immune cell responses. In addition, WAT contains beige adipocytes that have been shown to be key regulators of energy expenditure and the development of obesity. In the lean state, WAT is populated by type 2 cytokine-associated immune cells including AAMacs, eosinophils, ILC2s, Th2, and iNKT cells as well as anti-inflammatory cells such as T_{regs}. These immune cells participate in a complex dialog to maintain optimal immune and adipocyte function (Figure 2). Although the precise mechanisms by which type 2 immune cells in WAT regulate each other, it appears that elicitation of these cell pathways is associated with increased insulin sensitivity, optimal adipocyte mitochondrial function and in some cases elicitation of beige adipocytes within WAT (Figure 3). Conversely, disruption of these immunologic pathways results in impaired adipocyte function characterized by insulin resistance, oxidative stress, impaired respiratory capacity, and triglyceride deposition resulting in adipocyte hypertrophy and weight gain. Therefore, type 2 immune pathways in WAT appear to have protective roles that support maintenance of metabolic homeostasis and limit the development of obesity. This implies that eliciting type 2 immune cell pathways may be a useful strategy to treat or prevent obesity.

However, in the context of obesity, the immunologic milieu of WAT undergoes a dramatic shift from a type 2 to type 1 cytokine-associated inflammatory environment (Figure 4). Type 2 immune cells are decreased or dysregulated (e.g., ILC2s and eosinophils), and in some cases acquire a pro-inflammatory phenotype (e.g., iNKT cells and AAMacs). As type 2 immune cells tend to be associated with protection against obesity, these alterations in type 2 cytokine-associated immunologic pathways may contribute to the development of obesity and subsequent type 1 inflammatory responses. In addition, in obesity there is recruitment of various granulocytes, monocytes, and lymphocytes to WAT. These cell types produce cytokines such as TNF-α, IFN-γ, and IL-1β among others that potentiate type 1 immune responses and enhance antigen presentation to $CD4^+$ T cells, polarizing these cells toward a Th1 cell phenotype. In turn, Th1 cells produce additional TNF-α and IFN-γ, establishing a positive-feedback loop resulting in chronic low-grade type 1 inflammation and dysregulated glucose homeostasis. The precipitating factors that initiate type 1 immune responses in WAT are not well understood but may be related to adipocyte cell death (Spalding et al., 2008), hypoxia (Lee et al., 2014b; Sun et al., 2011), the generation of toxic lipid species (Muoio and Newgard, 2006), direct effects of dietary lipids or carbohydrates (Calder, 2002), and translocation of commensal bacteria to WAT (Amar et al., 2011; Cani et al., 2007) among other factors. The apparent multifactorial nature of the type 1 immune response in WAT suggests that targeting downstream inflammatory mediators such as IFN-γ, TNF-α, or IL-1β might have beneficial therapeutic effects in obesity-associated insulin resistance.

Finally, emerging studies have revealed complex immunomodulatory cross-talk between the type 1 and type 2 immune systems, where type 2 inflammation impairs type 1 responses and vice versa (Osborne et al., 2014; Reese et al., 2014; Stelekati and Wherry, 2012). Given the complex, dramatic shift in the immunologic landscape within obese WAT from a type 2 to type 1 cytokine-associated response, targeting a single immunologic factor may not be sufficient to treat obesity and restore

a normal immunologic profile in WAT. Clinical trials employing biologic agents that inhibit IL-1 (anakinra, canakinumab) or TNF-α (infliximab) in patients with type 2 diabetes or metabolic syndrome have been shown to produce moderate or no improvements in glucose metabolism, as assessed by glycated hemoglobin levels or indices of insulin resistance (Larsen et al., 2007; Ridker et al., 2012; Wascher et al., 2011). However the effects of these therapies on body weight or adiposity is not clear. Further research on immunomodulatory biologic therapy to treat obesity should be considered. In addition, the effectiveness of "two-factor" immunomodulatory therapies (e.g., neutralizing TNF-α antibody plus recombinant IL-33) should be explored as potential anti-obesity regimens. Targeting the immune system with biologics could represent a new strategy to limit food intake and/or increase energy expenditure to treat or prevent obesity and related metabolic diseases such as type 2 diabetes. In addition, understanding how current and future treatments for obesity (e.g., diet, exercise, drugs, and surgery) influence the immune system will be important for understanding their mechanisms of action and the potential side effects of treatment. Therefore, a deeper understanding of how the immune and metabolic systems interact to support metabolic homeostasis will be critical for understanding the biology of obesity and for the development of novel treatment and prevention strategies against this disease.

ACKNOWLEDGMENTS

The authors wish to thank members of the Artis laboratory for the critical reading of this manuscript. Research in the Artis lab is supported by the NIH (AI061570, AI074878, AI095466, AI095608, AI102942, AI106697, and AI097333 to D.A.), the Burroughs Wellcome Fund Investigator in Pathogenesis of Infectious Disease Award (D.A.) and Crohn's and Colitis Foundation of America (D.A.). Additional funding was provided by NIH F30-AI112023 and T32-AI060516 to J.R.B.

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Cell

A Century of Cholesterol and Coronaries: From Plaques to Genes to Statins

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http://dx.doi.org/10.1016/j.cell.2015.01.036

One-fourth of all deaths in industrialized countries result from coronary heart disease. A century of research has revealed the essential causative agent: cholesterol-carrying low-density lipoprotein (LDL). LDL is controlled by specific receptors (LDLRs) in liver that remove it from blood. Mutations that eliminate LDLRs raise LDL and cause heart attacks in childhood, whereas mutations that raise LDLRs reduce LDL and diminish heart attacks. If we are to eliminate coronary disease, lowering LDL should be the primary goal. Effective means to achieve this goal are currently available. The key questions are: who to treat, when to treat, and how long to treat.

Introduction

Among adults in industrialized countries, one-fourth of all deaths result from arterial blockage caused by atherosclerotic plaques (Heron, 2013). Most of these deaths are attributable to occlusion of the coronary arteries, which produces heart attacks. Against this background, it is quite surprising that heart attacks were recognized as clinical entities a mere 100 years ago. Within 50 years, the disease had increased to epidemic proportions. Only in recent years has the incidence of heart attacks in the United States begun to decline (Levy, 2012).

The multifaceted path by which cholesterol became linked to coronary heart disease is one of the great biomedical stories of the 20th century. Table 1 shows major events in the century of cholesterol and coronaries. It summarizes the scientific evidence that converged from multiple disciplines to implicate cholesterol-carrying low-density lipoprotein (LDL) as the instigator of atherosclerotic plaques and high dietary fat as the major cause of pathologic LDL levels. We have divided the discoveries into two sequential eras—first, the era of cholesterol and then the era of LDL.

This Review focuses on selected discoveries that we consider to be milestones in the century of cholesterol and coronaries. To cover 100 years of a robust field in a 6,000-word essay, we were forced to limit the literature that could be cited. We recognize the important contributions of many uncited scientists whose work deepened our understanding of cholesterol, LDL, and coronary atherosclerosis. The reader should be aware that our discussion of the SREBP pathway draws conclusions about human liver physiology that represent extrapolations from experiments in rats, mice, hamsters, and dogs. Inasmuch as measurements of similar depth cannot be made on the livers of human subjects, we believe that these extrapolations are justified by their explanatory potential for clinical and epidemiologic observations.

Cholesterol-Rich Plaques in Humans and Rabbits

Atherosclerotic plaques on the surface of the aorta were described by German pathologists in the 19th century (Leibowitz, 1970). The word atherosclerosis is derived from the Greek word *atheros*, which means gruel. It describes the cheesy substance that exudes from the plaques on sectioning. The first hint that the cheesy substance was cholesterol came in 1910 when the German chemist Adolf Windaus found that plaques from human aortas contained 25-fold more cholesterol than normal aortas. Shortly thereafter, in 1913, Nikolaj Anitschkow, a Russian pathologist, fed pure cholesterol to rabbits and produced profound atherosclerosis, thereby raising the possibility that dietary cholesterol is the source of the gruel (Anitschkow and Chalatow, 1913; Steinberg, 2005).

Heart Attacks Diagnosed in Living Patients

Although pathologists recognized that coronary arteries could be occluded by atherosclerotic plaques, this was considered to be a universally fatal event. Transient episodes of non-fatal chest pain were attributed to indigestion or other non-cardiac causes. This dogma was overturned in 1919, when the American clinician James Herrick used the recently invented electrocardiograph machine to demonstrate changes in the electrical pattern of the heart in patients who were experiencing severe non-fatal chest pain (Herrick, 1919, 1944). By providing a method for the firm diagnosis of heart attacks, the electrocardiogram ushered in the modern era of cardiology.

Familial Hypercholesterolemia Described

The connection between plasma cholesterol and heart attacks was put on firm genetic footing in 1938, when a Norwegian physician, Carl Müller, described families in which high plasma cholesterol levels were transmitted as an autosomal dominant trait (Müller, 1938). The disease was named familial hypercholesterolemia (FH), and it was soon recognized that the elevated cholesterol levels were associated with a 20-fold increase in the incidence of heart attacks in middle age.

First Description of Cholesterol Feedback

In 1933, Rudolph Schoenheimer, then working in Germany, placed mice in sealed bottles, fed them a cholesterol-free diet,

Table 1. A Century of Cholesterol and Coronaries

First Half—The Era of Cholesterol	
1910	Human atherosclerotic plaques contain cholesterol
1913	High cholesterol diet causes atherosclerosis in rabbits
1919	Heart attacks recognized in humans
1933	Feedback inhibition of cholesterol synthesis demonstrated
1938	Familial hypercholesterolemia described
1950	Cholesterol biosynthetic pathway elucidated
1951	High-fat diets raise plasma cholesterol in humans
1953	Risk factor concept advanced
Second Half—The Era of LDL	
1955	LDL identified as risk factor for CHD
1973	LDL receptor discovered
1976	HMG CoA reductase inhibitors (statins) discovered
1981	Statins increase LDL receptors in vivo
1987	First statin (Mevacor) approved for human use
1994	Statins decrease heart attacks and prolong life
1997	SREBP pathway elucidated
2006	PCSK9: Destroyer of LDL receptors

and found that the cholesterol content of the bottles increased. When the diet contained cholesterol, there was no longer a contribution from the mice. This landmark study demonstrated not only that animals can synthesize cholesterol, but also that synthesis is inhibited when cholesterol is present in the diet (Schoenheimer and Breusch, 1933). This was the first demonstration of the fundamental principle of end-product feedback inhibition of a biosynthetic pathway, pre-figuring the classic work of Jacob and Monod (1961), Pardee and Reddy (2003), and Umbarger (1992). Schoenheimer's discovery also laid the groundwork for the discovery of the LDL receptor in the 1970s (Goldstein and Brown, 2009) and the Scap/SREBP pathway in the 1990s (Brown and Goldstein, 2009).

Cholesterol Biosynthetic Pathway Elucidated

In the 1950s, a small group of talented biochemists worked out the complex pathway by which the 27-carbon, 4-ring cholesterol molecule is synthesized through repeated polymerizations from acetate, a simple 2-carbon building block attached to CoA. Most prominent in this endeavor were Konrad Bloch and Feodor Lynen, who shared the 1964 Nobel Prize in Physiology or Medicine for their discoveries (Bloch, 1965; Zetterström, 2009). Of particular importance was the identification by Bucher and Lynen of the 6-carbon compound 3-hydroxy-3-methylglutarate attached to CoA (HMG CoA) as the first intermediate committed solely to synthesis of cholesterol and other isoprenoids (Bucher et al., 1960). This finding focused attention on HMG CoA reductase as the rate-controlling enzyme in the cholesterol biosynthetic pathway.

Risk Factor Concept Advanced

In 1951, Paul Dudley White and his colleague, Menard M. Gertler, cardiologists at the Massachusetts General Hospital in Boston, made careful observations on 100 people under age 40 who had suffered heart attacks. They identified a series of risk factors that predisposed to the disease (Gertler and White, 1954). These consisted of male sex, high blood cholesterol, elevated blood pressure, cigarette smoking, positive family history, and a mesomorphic body build. All of these are recognized as risk factors today.

Seven-Country Study Invokes Dietary Fat

In 1953, the American physiologist Ancel Keys launched a landmark international epidemiological study of heart attacks. Keys (1980) chose to study 16 cohorts of healthy men from specific populations in seven different countries. The cohorts were chosen mainly because their diets contained very different amounts of total and saturated fat—ranging from minimal fat in a cohort of Japanese fishermen who thrived on vegetables, rice, and fish to enormous amounts of fat in East Finnish foresters who habitually spread butter on their cheese. For each of the 16 cohorts, Keys ascertained 511 to 2,517 healthy men between the ages of 40 and 59 and followed them for 10 years, recording fatal and non-fatal heart attacks (Keys, 1980). He found that serum cholesterol rose in proportion to the total fat intake ($r = 0.67$) and even more strikingly in proportion to the intake of saturated fatty acids ($r = 0.87$). Fatal coronary events rose in proportion to the serum cholesterol level ($r = 0.80$). At the two extremes, serum cholesterol varied from a mean of 165 mg/dl in the Japanese fishermen to 270 mg/dl in the East Finnish foresters. This difference was accompanied by a 13-fold higher incidence of coronary events in the East Finnish. The other 14 cohorts lay more or less on a line relating dietary saturated fat intake and serum cholesterol. Although certain deviations from this line have been proposed to contradict Keys' conclusion, it is likely that the deviations reflect local environmental or genetic factors that modify the relation between fat intake, serum cholesterol, and heart attacks in specific populations. However, they do not negate Keys' general conclusion that high fat intake leads to high cholesterol and to heart attacks.

Keys' observation that low dietary fat produces low blood cholesterol and low heart attacks was supported by comparative studies of Japanese men living in Japan, Hawaii, and California (Keys et al., 1958). As the Japanese moved westward, their fat intake increased, their plasma cholesterol rose, and the number of heart attacks increased. Keys' studies were performed before plasma lipoproteins could be readily fractionated, and we therefore consider them to conclude the first era in the story of coronaries—the era of cholesterol.

LDL Identified as Major Risk Factor

The era of LDL began in 1955, when John Gofman, a physician/physicist at the University of California, Berkeley, used the newly invented analytical ultracentrifuge to separate the cholesterol-carrying lipoproteins of plasma according to their density (Gofman et al., 1954a). Two major fractions were identified: low-density lipoproteins (LDL) and high-density lipoproteins (HDL). When he studied plasma from heart attack patients, Gofman found a major increase in the cholesterol-carrying LDL. He also observed a reduced level of HDL (Gofman et al., 1954b). Gofman's pioneering discoveries have been replicated many times. The correlation between high LDL levels, low HDL levels, and heart attacks is one of the most profound epidemiologic correlations in all of medicine.

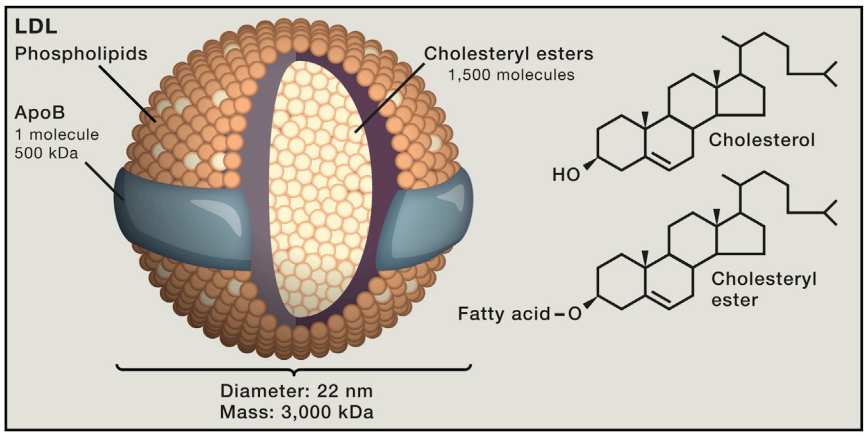

Figure 1. LDL, A Cholesterol Carrier
LDL is a spherical particle with a diameter of 220 nm and a mass of ~3,000 kDa. Each particle contains ~1,500 molecules of cholesteryl ester in an oily core that is shielded from the aqueous plasma by a hydrophilic coat composed of ~800 molecules of phospholipid, ~500 molecules of unesterified cholesterol, and 1 molecule of a 500 kDa protein, apoB.

Gofman's finding of a positive correlation with LDL led to intense interest in this pathologic lipoprotein (Fredrickson et al., 1967). In contrast, his finding of a negative correlation with HDL received little attention until it was rediscovered 20 years later by epidemiologists, most notably in the Framingham study (Gordon et al., 1977). Despite the enormous amount of data correlating low HDL levels with increased heart attacks, the mechanistic role of HDL in the pathogenesis of coronary disease is poorly understood and remains controversial despite 40 years of study. In marked contrast, elevations of plasma LDL have been shown to produce atherosclerosis in every mammalian species ever studied.

Figure 1 shows the structure of LDL. Each LDL particle contains ~1,500 molecules of cholesteryl ester in a hydrophobic core surrounded by a polar phospholipid coat and a single large protein called apolipoprotein B (apoB). A prevalent theory states that LDL initiates atherosclerotic plaques when it penetrates through dysfunctional endothelium into the walls of arteries (Bonetti et al., 2003). LDL is retained there, likely by its propensity to bind to glycosaminoglycans. The lipids of LDL become oxidized, and some of the reaction products modify lysine residues on apoB (Steinberg and Witztum, 2010). The modified apoB is recognized by scavenger receptors on macrophages and internalized by endocytosis, converting the macrophages to cholesterol-laden foam cells (Brown and Goldstein, 1983; Greaves and Gordon, 2009). The foam cells secrete a variety of cytokines, thereby initiating an inflammatory reaction (Hansson and Jonasson, 2009; Libby et al., 2011). In response, smooth muscle cells in the arterial intima proliferate and produce collagen. The plaque enlarges and eventually ruptures, leading to the formation of a blood clot that occludes the vessel.

All other things being equal, the higher the LDL the faster the plaques evolve. However, all other things are not equal. At any given level of LDL, plaque formation is accelerated by risk factors that include smoking, high blood pressure, and diabetes. In addition, poorly understood genetic factors affect atherosclerosis, possibly by changing the susceptibility of the endothelium to become damaged. Unless the LDL level is extraordinarily high or low, it is difficult to predict accurately whether any individual will suffer a heart attack.

LDL Receptor Pathway Elucidated

In 1972, we entered the field when we began to study the regulation of cholesterol synthesis in cultured human fibroblasts (Goldstein and Brown, 2009). We were stimulated by our encounter with two young siblings (ages 6 and 8) who were hospitalized at the National Institutes of Health because of recurrent heart attacks, owing to plasma LDL levels that were 8-fold above normal. The children had the severe homozygous form of familial hypercholesterolemia (FH), the disease that was described in 1938 in its common heterozygous form by Müller in Norway (see above) and later in its rare homozygous form by Khachadurian (1964) in Lebanon. In the homozygous children, each LDL particle was structurally normal, but they had 8-fold more particles per ml of plasma as compared with normal children. Homozygous FH is a rare disorder with a frequency of ~1 in 1 million people (Goldstein et al., 2001).

Frustrated at being unable to reduce the LDL level in these unfortunate children, we resolved to elucidate the genetic defect. In 1972, shortly after moving to the University of Texas Southwestern Medical Center in Dallas, we obtained skin biopsies from three FH homozygotes and controls. From the biopsies, we grew monolayers of fibroblasts. Like all human cells, cultured fibroblasts require cholesterol to maintain the integrity of their plasma membranes. We found that normal fibroblasts obtained cholesterol from two sources (Figure 2A). They synthesized it from acetyl CoA through the pathway described by Bloch, Lynen, and others, including the rate-controlling step catalyzed by HMG CoA reductase. In addition, normal fibroblasts somehow took up cholesterol from the LDL in the serum of the culture medium. In sharp contrast, cells from FH homozygotes could not take up cholesterol from LDL. They survived in culture because they activated the cholesterol synthesis pathway to compensate for the lack of LDL-derived cholesterol (Figure 2B). Their HMG CoA reductase activity was 100-fold above normal (Goldstein and Brown, 1973).

Working intensely for several years, we found the mechanism for LDL uptake. The key is a cell surface receptor that binds apoB. With our colleague Richard Anderson, we showed that LDL receptors cluster in coated pits, regions of the cell surface that are adapted for rapid internalization by endocytosis (Anderson et al., 1977; Goldstein et al., 1979). The internalized LDL is delivered to lysosomes where the cholesteryl esters are hydrolyzed, and the cholesterol is released for new membrane synthesis. We also found that the production of LDL receptors and the enzyme HMG CoA reductase are subject to coordinate feedback suppression, as indicated by the dashed arrows in Figure 2A.

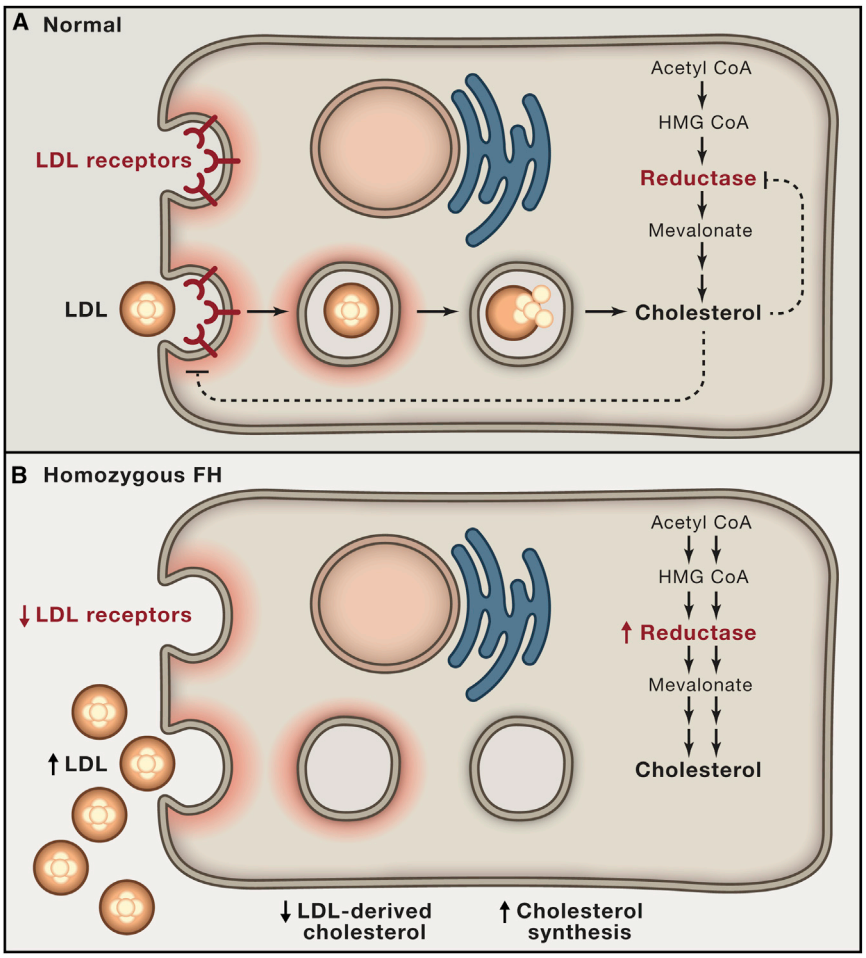

Figure 2. Feedback Regulation of Cholesterol Synthesis and LDL Receptors in Cultured Cells from Normal Subjects and Children with Homozygous FH

(A) Normal cells obtain cholesterol from two sources: (1) endogenous synthesis and (2) receptor-mediated uptake and lysosomal hydrolysis of LDL.

(B) Lacking LDL receptors, FH cells maintain normal levels of cholesterol by increasing synthesis of cholesterol, leaving excess LDL in the culture medium.

When cell cholesterol levels are low, the cells produce abundant LDL receptors and HMG CoA reductase. When cellular cholesterol levels rise, the levels of these two proteins decrease. The net result is to keep the level of cholesterol in cell membranes constant (Brown and Goldstein, 1986).

Defective LDL Receptors Cause FH

We found that the defect in FH lies in the gene for the LDL receptor (Brown and Goldstein, 1974). Depending on the site of the mutation in the LDL receptor gene, some FH homozygotes are unable to produce any functional receptors (Figure 2B), whereas others produce receptors with 5%–25% of normal activity (Goldstein et al., 2001). FH heterozygotes produce half the normal number of functional receptors. Their LDL levels are elevated by ~2-fold, and they have heart attacks in their fifth and sixth decades. The heterozygous form of FH is relatively common for a single-gene disease, occurring in at least 1 in 500 people in all populations (Goldstein et al., 2001).

LDL is secreted from the liver as a larger particle called very low-density lipoprotein (VLDL) that contains triglyceride as well as cholesterol. The triglycerides are extracted in adipose tissue and muscle, causing the particle to shrink and become LDL. Most LDL is removed from the circulation by LDL receptors located in the liver. When LDL receptors are deficient, LDL particles circulate for a prolonged time and build up to high levels in plasma, eventually depositing in arteries and thereby creating atherosclerotic plaques (Brown and Goldstein, 1986).

Our laboratory purified the LDL receptor from bovine adrenal glands, which use the receptor to supply cholesterol for synthesis of steroid hormones (Schneider et al., 1982). We obtained a partial amino sequence and used it to clone the cDNA and the gene (Yamamoto et al., 1984; Südhof et al., 1985). Located on the short arm of chromosome 19, the 18 exons of the LDL receptor gene encode a mature protein of 839 amino acids. The receptor spans the plasma membrane with its NH_2 terminus facing the exterior. The external segment contains seven cysteine-rich repeats that constitute the LDL-binding site. The cytoplasmic COOH-terminal segment of 50 amino acids contains the sequence that guides the receptor to coated pits (Chen et al., 1990). To date, more than 1,200 different allelic mutations in the LDL receptor gene have been described in different families with FH (Usifo et al., 2012). Some mutations destroy all function, while others allow some residual activity.

Statins Inhibit HMG CoA Reductase and Increase LDL Receptors

A therapeutic milestone occurred in 1976, when Akira Endo at the Sankyo Company in Tokyo identified the first inhibitor of HMG CoA reductase, thereby inaugurating the class of cholesterol-lowering drugs known as statins (Endo et al., 1976). Endo's statin, called compactin, was isolated from a penicillium mold. Knowing about the feedback suppression of LDL receptors, we reasoned that an HMG CoA reductase inhibitor would deprive liver cells of endogenous synthesis as a source of cholesterol. This deprivation would relieve the feedback repression of LDL receptors, and the resultant increase in LDL receptors would lower plasma LDL (Brown et al., 1978). In 1981, we tested this hypothesis by treating dogs with mevinolin, another fungal HMG CoA reductase inhibitor and closely related to

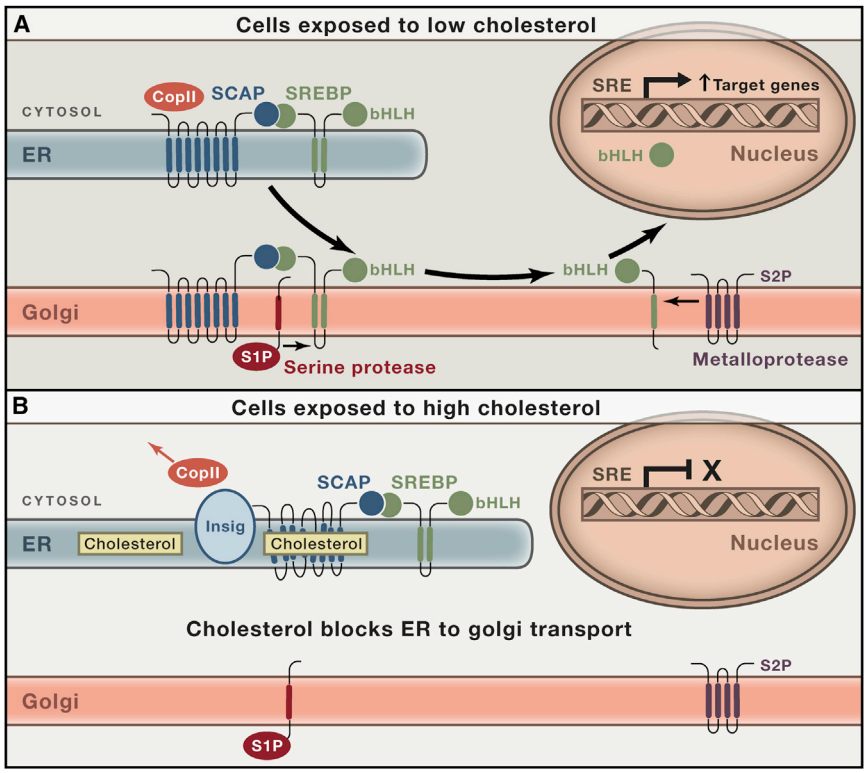

Figure 3. The SREBP Pathway for Cholesterol Homeostasis in Animal Cells

(A) Cholesterol deficiency. When the cholesterol content of ER membranes falls below 5 mol% of its total lipids, Scap binds COPII proteins, which incorporate the Scap/SREBP complex into COPII-coated vesicles that move to the Golgi. In the Golgi, SREBPs are cleaved by two proteases, S1P and S2P, allowing the active portion to enter the nucleus, where it activates genes that increase cholesterol synthesis and uptake.

(B) Cholesterol excess. When ER cholesterol rises above 5 mol% of membrane lipids, cholesterol binds to Scap, thereby causing Scap to bind to Insig. This releases COPII proteins, abrogating transport to the Golgi. The fall in nuclear SREBP reduces transcription of genes for cholesterol synthesis and uptake.

compactin, which was discovered by Alfred W. Alberts and colleagues at Merck (Alberts et al., 1980). As predicted, mevinolin increased LDL receptors in the dog liver, and this led to a marked fall in plasma LDL (Kovanen et al., 1981). Subsequent studies by others showed that mevinolin (soon known as lovastatin or Mevacor) and other statins lowered plasma LDL in humans with diet-induced hypercholesterolemia (Vega and Grundy, 1991) and also in FH heterozygotes whose single functional LDL receptor gene is susceptible to activation by cholesterol deprivation (Mabuchi et al., 1981). Consistent with the LDL receptor theory, those FH homozygotes who had no functional receptors showed no cholesterol lowering when treated with mevinolin (Uauy et al., 1988).

First Statin Approved for Lowering LDL

In 1987, Merck's Mevacor became the first statin approved for human use (Brody, 1987; Byrne, 1987). The FDA approved Mevacor based on studies showing that it lowers plasma LDL and is well tolerated. At the time of approval, there was no evidence that a statin could reduce heart attacks. This evidence came in 1994, when Merck's 4S Study (Scandinavian Simvastatin Survival Study Group, 1994) demonstrated that a second-generation statin, simvastatin, not only reduced heart attacks, but actually prolonged life in middle-aged people at high risk of a coronary event.

SREBP Discovered

The statin studies showed that the feedback regulation of LDL receptors is of clinical importance, yet nothing was known of the molecular mechanism by which cholesterol regulated the synthesis and supply pathways. Essentially all cellular cholesterol is contained in membranes. The feedback system optimizes the level of cholesterol in those membranes. The puzzle is: How does a cell measure the level of cholesterol in its membranes, and how is this information transmitted to the nucleus to regulate gene transcription? The first clue came in 1993, when we isolated the key regulator—a transcription factor called sterol regulatory element-binding protein-1 (SREBP-1) (Yokoyama et al., 1993).

We purified SREBP-1 from the nuclei of cultured cells that were deprived of cholesterol so that they actively transcribed the LDL receptor gene. Our isolation procedure was based on the ability of the protein to bind to a DNA sequence required for LDL receptor transcription (Wang et al., 1993). The purified protein had a molecular mass of ~60 kDa; yet when we cloned its cDNA, we were greeted with two surprises. First, the protein purified from nuclei was only a fragment of the full-length SREBP-1, which had a predicted molecular mass of 125 kDa (Yokoyama et al., 1993). And second, the newly synthesized protein was bound to membranes by virtue of two transmembrane helices (Wang et al., 1994; Hua et al., 1995). We were confronted with a problem: how does a membrane-bound transcription factor move to the nucleus?

SREBP Pathway Elucidated

Over the next few years, we solved the movement problem, and the result is the SREBP pathway shown in Figures 3A and 3B (Brown and Goldstein, 1997; Goldstein et al., 2006). SREBPs are synthesized as integral membrane proteins of the endoplasmic reticulum (ER). The cytosolic NH_2-terminal segment (molecular mass, 60 kDa) contains the basic helix-loop-helix-leucine zipper sequence by which the protein binds to DNA and activates transcription. Next is a hairpin membrane-attachment domain comprising two transmembrane helices separated by a 50 amino acid loop that projects into the ER lumen. At the COOH-terminal end is a 65 kDa regulatory domain that projects into the cytosol.

Immediately after its translation on membrane-bound ribosomes, SREBP binds to Scap, a polytopic protein of the ER membrane (Hua et al., 1996). Scap has eight transmembrane helices and a COOH-terminal extension that projects into the cytosol, where it binds to the COOH-terminal regulatory domain of SREBP (Sakai et al., 1998a). The cytosolic loop between helices 6 and 7 of Scap contains a sequence of six amino acids that binds to a complex of proteins called COPII (Sun et al., 2007), which were known previously to cluster certain ER proteins into COPII-coated vesicles that bud from ER membranes (Antonny and Schekman, 2001). These vesicles transport the Scap/SREBP complex to the Golgi apparatus.

In the Golgi, SREBP is processed sequentially by two proteases. The first, called site-1 protease (S1P), is a membrane-bound serine protease that clips the SREBP in the luminal loop (Sakai et al., 1998b). Cleavage separates the SREBP into two membrane-bound halves. The NH_2-terminal segment remains bound to the membrane, owing to its single membrane-spanning helix. It is released from the membrane by site-2 protease (S2P), a hydrophobic zinc metalloprotease with its active-site zinc buried in the membrane (Rawson et al., 1997; Feng et al., 2007). S2P cleaves the NH_2-terminal half of SREBP within its membrane-spanning helix, releasing the transcriptionally active segment so that it can enter the nucleus to activate transcription of the LDL receptor gene. Intramembrane zinc metalloproteases related to S2P are found as far back as archaea. We coined the term regulated intramembrane proteolysis (RIP) to designate the process by which membrane-bound proteins are released by cleavage within a transmembrane helix (Brown et al., 2000). Multiple examples of this regulatory mechanism are now known (Urban, 2013).

Scap: A Sterol Sensor

Its location in the ER membrane allows Scap to sense the level of cholesterol in the membrane (Radhakrishnan et al., 2004, 2008). When ER cholesterol rises above a threshold of 5% of total lipids, the cholesterol binds to luminal loop 1 of Scap (Motamed et al., 2011). This binding triggers a conformational change in luminal loop 6 (Brown et al., 2002) that occludes the binding site for COPII proteins (Sun et al., 2007). In its new conformation, Scap binds to Insig, another polytopic protein of ER membranes (Yang et al., 2002). Insig locks Scap into a conformation that cannot bind COPII proteins, trapping the Scap/SREBP complex in the ER, preventing Golgi processing, and thereby decreasing transcription of the LDL receptor gene. Transcription remains low until ER cholesterol falls and cholesterol-free Scap/SREBP complexes can again bind COPII proteins.

Although SREBP-1 was isolated by virtue of its ability to activate transcription of the LDL receptor gene, we soon found that the protein activates the gene for HMG CoA reductase as well as the genes for all of the enzymes in the cholesterol biosynthetic pathway, thereby allowing cells to synthesize cholesterol in addition to taking it up from LDL (Shimano et al., 1996; Horton et al., 2003). SREBP-1 has two isoforms, SREBP-1a and -1c, which are derived from alternate promoters that give rise to different first exons (Shimomura et al., 1997). SREBP-1a is highly expressed in rapidly growing normal and cancer cells; it activates fatty acid synthesis as well as cholesterol synthesis, thereby supplying the major lipid components of cell membranes. SREBP-1c is expressed primarily in liver, where it activates fatty acid synthesis in response to insulin (Shimomura et al., 1999), thereby contributing to fatty liver in type 2 diabetes (Moon et al., 2012). A third isoform, SREBP-2, is produced by a different gene. It is a relatively specific activator of the genes encoding the LDL receptor and cholesterol biosynthetic enzymes (Horton et al., 2003). All three SREBPs require processing by the SREBP pathway (Horton et al., 2002).

Regulation of LDL Receptors by Diet, Drugs, and Genes

Figure 4 shows a model to explain how the regulation of SREBP-2 in the liver is responsible for the LDL elevation produced by high-cholesterol/high-fat diets and the LDL-lowering produced by statins. The model is based on experiments in hamsters and mice (Sheng et al., 1995; Horton et al., 2002). When the diet is low in cholesterol, liver cholesterol levels are low and SREBP-2 enters the nucleus, where it activates the LDL receptor gene, the HMG CoA reductase gene, and other genes required for cholesterol synthesis (Figure 4A). The LDL receptors keep plasma LDL low. High cholesterol diets cause cholesterol to accumulate in hepatic ER membranes, blocking SREBP-2 processing, reducing LDL receptors, and raising plasma LDL (Figure 4B). In humans, the diet-mediated receptor reduction is not complete or everyone would look like FH homozygotes. It is not even 50% as in FH heterozygotes. However, it is sufficient to raise LDL from the low levels seen in Japanese fishermen to the higher levels accepted as normal in the U.S. and other industrialized countries. Statins have the opposite effect. By blocking cholesterol synthesis, statins lower ER cholesterol, activating SREBP-2, increasing LDL receptors, and lowering plasma LDL (Figure 4C). Nuclear SREBP-2 also increases HMG CoA reductase, but cholesterol synthesis does not increase, owing to statin inhibition. Strongly in favor of the receptor induction mechanism is the observation that statins do not lower LDL significantly in those FH homozygotes who have null mutations in both copies of the LDL receptor gene (Uauy et al., 1988).

Figure 5 illustrates the effects of diet, drugs, and genes on plasma LDL and the consequent effects on coronary disease. The top panel (Figure 5A) is a graphic representation of plasma cholesterol levels in the human species as extrapolated from surveys of middle-aged people in major populations of the world. We use total cholesterol values because there are more measurements of total cholesterol than of LDL. However, differences in total cholesterol closely reflect differences in LDL. The distribution of total cholesterol levels is highly skewed. The data predict that the median cholesterol level for the human species is ~170 mg/dl. This is attributable to the large number of people in China and other Asian populations, where the historical mean cholesterol level is about 150 mg/dl (Chen et al., 1991; Junshi et al., 1990). In North America and Europe, the mean value is ~220 mg/dl. The incidence of heart attacks correlates with these levels. Almost unheard of in China and Japan before recent increases in their fat intake, heart attacks are much more frequent in Europe and the U.S., especially when plasma cholesterol exceeds 220 mg/dl (graduated red shading in Figure 5A).

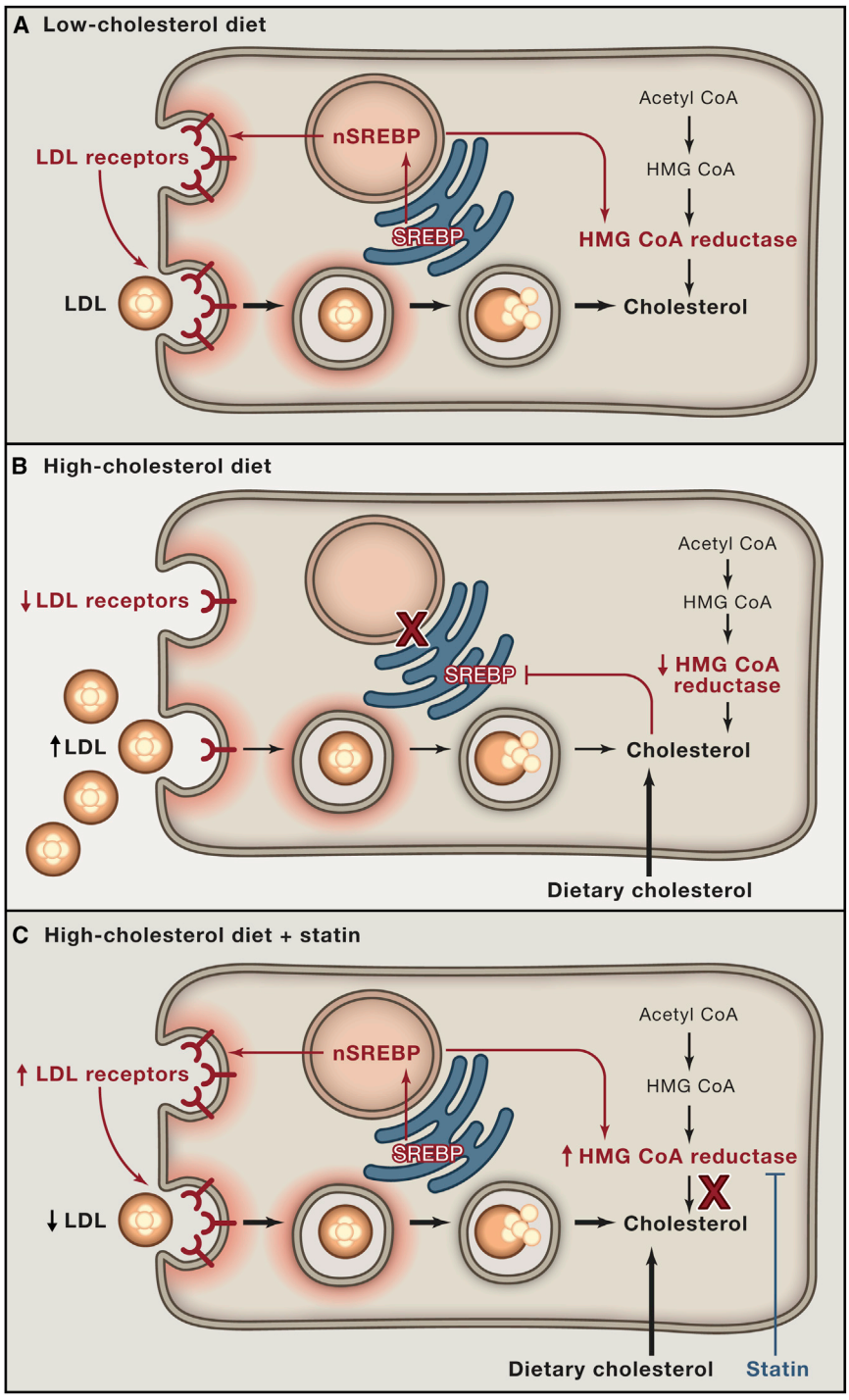

Figure 4. Hepatic Response to Diet and Statins Mediated by the SREBP Pathway

(A) Low-cholesterol diet. Proteolytic cleavage of SREBP is increased. The cleaved SREBP enters the nucleus to activate genes controlling cholesterol synthesis (including HMG CoA reductase) and uptake (LDL receptor). nSREBP, nuclear portion of cleaved SREBP.

(B) High-cholesterol diet. Proteolytic cleavage of SREBPs is decreased, resulting in decreased nuclear SREBP and decreased activation of target genes. The decrease in LDL receptors produces an increase in plasma LDL.

(C) High-cholesterol diet plus statin therapy. Statins inhibit HMG CoA reductase, causing a transient decrease in ER cholesterol. In response, SREBP cleavage is increased, and the resulting nuclear SREBP activates the genes for HMG CoA reductase and LDL receptor. The increased HMG CoA reductase is inhibited by the statin, and the increased LDL receptors lower plasma LDL.

Statins Reduce Heart Attacks and Prolong Life

Figure 5B summarizes the results of four double-blind, placebo-controlled trials in which middle-aged people at high risk for heart attacks were treated for 5 years with a statin or placebo (38,153 total subjects). The results are remarkably consistent. In each case, lowering LDL reduced heart attacks with no evidence for a lower threshold (Scandinavian Simvastatin Survival Study Group, 1994; Sacks et al., 1996; LIPID, 1998; Heart Protection Study Collaborative Group, 2002). In the largest study, the Heart Protection Study (HPS), 20,536 subjects were treated with simvastatin for 5 years. The study was large enough to allow multiple subgroup analyses. These showed that the reduction in relative risk was similar even when subjects had risk factors, including diabetes, hypertension, and smoking. A recent meta-analysis of 22 statin trials involving 134,000 participants concluded that, for each reduction of LDL cholesterol of 1 mmol/l (40 mg/dl), cardiovascular events are reduced by 20%, even in people who were considered to be at low risk (Mihaylova et al., 2012). Considered together, these statin trial results strongly support the dominant role of LDL in coronary disease.

PCSK9: A Protein that Destroys LDL Receptors and Raises LDL

The genetic connection between LDL and heart attacks was established by mutations in LDL receptors that raise LDL and increase coronary events (Brown and Goldstein, 1986). Recently, the other side of the coin was exposed—namely, mutations that increase LDL receptors, lower LDL, and reduce coronary events (Cohen et al., 2006; Abifadel et al., 2014). The mutations occur in a gene that encodes an enzyme called PCSK9, which stands for proprotein convertase subtilisin/kexin type 9 (Horton et al., 2009). Secreted into plasma by liver and other organs, PCSK9 binds to hepatic LDL receptors and disrupts the recycling mechanism that returns the receptors to the cell surface after internalization (Lagace et al., 2006; Zhang

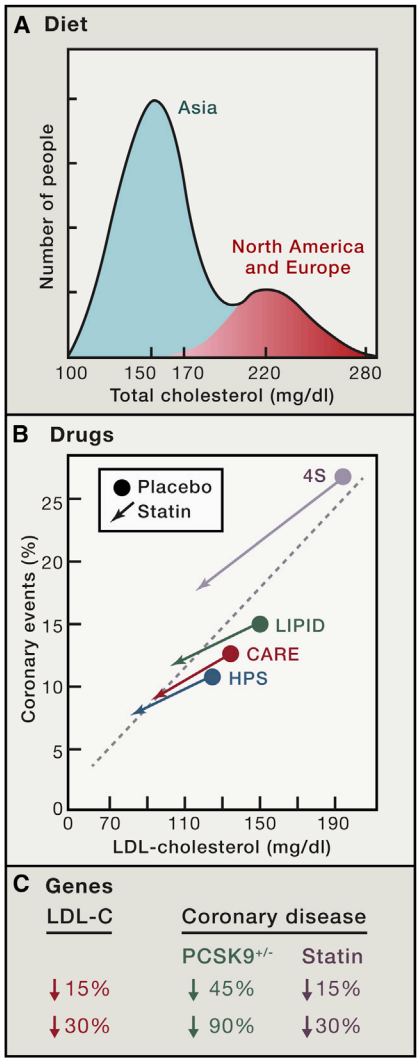

Figure 5. Diagram Illustrating the Effects of Diet, Drugs, and Genes on Plasma LDL and Coronary Disease

(A) Diet. Idealized depiction of the frequency distribution of plasma cholesterol levels in the human species as extrapolated from surveys of middle-aged people in major populations of the world. The higher the cholesterol level, the higher the risk for coronary disease, as denoted by the graded red shading.

(B) Drugs. Frequency of coronary events plotted against plasma level of LDL cholesterol in four double-blind, placebo-controlled trials in which middle-aged people at risk for heart attacks were treated for 5 years with a statin or placebo. The number of subjects in each study was as follows: 4S Study, 4,444; LIPID, 9,014; CARE, 4,159; and HPS, 20,536.

(C) Genes. Difference in risk for coronary disease in middle-aged people depending on whether plasma LDL cholesterol level is reduced over a lifetime (heterozygous loss of function of PCSK9) or for only 5 years (statin therapy).

et al., 2007). As a result, the number of cell-surface LDL receptors declines and LDL rises. Inasmuch as PCSK9 circulates in the plasma of normal individuals, plasma LDL is never as low as it would be if LDL receptors were functioning maximally.

The relation between PCSK9 and LDL receptors was discovered by Abifadel et al. (2003) in studies of rare patients with gain-of-function mutations that increase the activity of the protein. Affected individuals have high LDL levels that are transmitted as an autosomal-dominant trait. The reverse phenomenon was discovered by Cohen and Hobbs, who identified people with common loss-of-function mutations in PCSK9 that lead to low LDL levels and protect against heart attacks (Cohen et al., 2006). Three percent of Caucasians are heterozygous for a missense mutation (R46L) that partially inactivates PCSK9. On average, these individuals have a 15% reduction in plasma LDL when compared with age-matched controls. Remarkably, they have a 46% reduction in early coronary events. Even more striking, 2% of African-Americans are heterozygous for one of two nonsense mutations (either Y142X or C679X) that inactivate one copy of the PCSK9 gene. Their LDL levels are reduced by 28%, and they have an 88% reduction in heart attacks when studied at a mean age of 54 years (Cohen et al., 2006).

Cohen and Hobbs' finding of a 45% lowering of coronary events in Caucasians with missense mutations in PCSK9 was rapidly confirmed in two other studies (McPherson and Kavaslar, 2007; Kathiresan, 2008). The reduction in coronary disease in individuals with diminished PCSK9 occurs despite the presence of other risk factors, including hypertension, diabetes, and smoking. This finding correlates with the finding in the HPS study that LDL lowering with a statin can reduce heart attacks even when other risk factors are present (Heart Protection Study Collaborative Group, 2002).

LDL Reduction: Better Sooner Than Later

The observations on subjects with PCSK9 mutations suggest strongly that lifelong LDL reductions are much more effective than late LDL reductions in preventing coronary events. Figure 5C compares the reduction in coronary disease in PCSK9 heterozygotes and the reduction observed in people in 5-year clinical trials whose LDL was lowered by a statin. When statins lower LDL by 15%, they lower coronary events by 15%, which is much less than the 45% reduction observed when LDL is reduced to a similar degree by a PCSK9 mutation. Similarly, when statins lower LDL by 30%, they lower coronary events by 30%—far weaker than the nearly 90% event reduction observed when LDL is lowered by 30% as a result of a PCSK9 mutation. Why is the protective effect of the PCSK9 mutations so much greater than can be achieved by statins? The most likely explanation lies in the duration of the LDL lowering (Brown and Goldstein, 2006). People with PCSK9 mutations have low LDL levels throughout life, and they may never have developed atherosclerotic plaques. In the statin trials, patients were not treated until they were at high risk of a coronary event, which means that they already had established atherosclerosis. Once the disease is established, patients may require much more severe LDL lowering to prevent an event.

It is noteworthy that the 90% reduction in coronary events in people with null mutations in PCSK9 is similar to the 90% reduction observed by Ancel Keys when he studied Japanese fishermen who had low LDL levels throughout life (Keys, 1980). The advantage of lifelong low LDL is further illustrated by a recent study of subjects who absorb reduced amounts of cholesterol from the intestine as a result of loss-of-function mutations in NPC1L1, the intestinal cholesterol transporter. Lifelong reductions of only 11% in LDL produced a 53% reduction in heart

attacks (The Myocardial Infarction Genetics Consortium Investigators, 2014). Thus diet, genes, and drugs all yield the same conclusion, namely, that LDL lowering can prevent heart attacks, especially when the lowering is begun before atherosclerotic plaques have developed.

Based on the high affinity of the LDL receptor for LDL, in 1977 we postulated that the human body is designed to maintain LDL cholesterol levels in the range of 25 mg/dl (Goldstein and Brown, 1977, 1982). Indeed, plasma LDL cholesterol levels in adults from most other animals so far studied, including primates, are lower than 60 mg/dl (Mills and Taylaur, 1971; Chapman and Goldstein, 1976). In newborn humans, LDL cholesterol is less than 50 mg/dl (Kwiterovich et al., 1973). LDL levels above 100 mg/dl are frequent only in humans or other animals that have consumed the typical Western diet, which is high in fat and cholesterol.

The dramatic reductions in coronary events in people with PCSK9 mutations stimulated several pharmaceutical companies to produce monoclonal antibodies that bind to PCSK9 and inactivate it. Data from two early trials show that these antibodies can reduce plasma LDL by as much as 40% when given alone and by 60% when added to a statin regimen (Stein et al., 2012; Raal et al., 2012). Just like statins, anti-PCSK9 antibodies fail to reduce LDL significantly in those FH homozygotes who lack all LDL receptor activity (Stein et al., 2013), further supporting the role of LDL receptors in lowering LDL. Studies are underway to determine the long-term safety of anti-PCSK9 antibodies and their efficacy in preventing heart attacks (Young and Fong, 2012; Vogel, 2012).

Accomplices of LDL

The data reviewed here focus on LDL as the *sine qua non* of atherosclerosis. However, LDL does not act alone. Evidence from genetically manipulated mice indicates that the atherogenic effect of LDL requires macrophages and inflammatory cytokines (Glass and Witztum, 2001; Libby et al., 2011). In humans, at any given level of LDL, the probability of a myocardial infarction is increased by well-documented risk factors that include cigarette smoking, hypertension, diabetes, and low HDL. Although these contributory factors are important, they all require LDL to instigate the lesion. The epidemiologic data in China and Japan, the genetic data with PCSK9 and NPC1L1 deficiency, and the therapeutic data with statins demonstrate that myocardial infarctions can be markedly diminished by LDL lowering even when macrophages, cytokines, and risk factors are present. Fortunately, we now have the ability to lower LDL with diet and with drugs.

The Next Century

The past 100 years have witnessed the crest of the coronary disease epidemic in Western countries and its beginning recession. Scientists have made dramatic advances in understanding cholesterol, the lipoproteins that carry it in plasma, and the manner in which genes and diets alter lipoprotein levels. The century of cholesterol research produced four lines of evidence—experimental, genetic, epidemiologic, and therapeutic—that converge upon LDL as the primary cause of atherosclerosis. Few, if any, chronic diseases have been subjected to such intensive scrutiny, and rarely has the cause and the approach to prevention been documented so convincingly.

It does not seem an exaggeration to state that targeted application of an LDL-lowering regimen may eventually curtail one of the major killers of the last century. The key questions for the 21st century are who to target and when. Ideally, LDL-lowering therapy should be initiated before atherosclerotic plaques develop or at least before they develop their most threatening features. Currently, we base our decisions for LDL lowering on factors such as the absolute level of LDL, the presence of other risk factors, and a family history of early coronary disease. In the future, we may be able to use genetic polymorphisms that render arteries susceptible or resistant to LDL entry and susceptible or resistant to the ensuing inflammation. Such genetic polymorphisms may increase our assessment of risk, but they are unlikely to be absolute. Our decisions would be greatly helped if someone develops a noninvasive method to detect the earliest atherosclerotic plaques in the coronary arteries, to monitor their growth, and to detect the earliest signs of the inflammation and instability that lead to thrombosis. Given such a method for early detection and given our powerful methods to lower LDL, we should surely be able to end the epidemic of heart attacks that killed so many over the last century.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the many talented students, postdoctoral fellows, and collaborators who have conducted the experiments described from our laboratory. Their names are included in the cited references. Our experimental work over the last 40 years has been supported by grants from the NIH (HL-20948), the Perot Foundation, Moss Heart Foundation, and the O'Donnell Foundation. J.L.G. and M.S.B. are members of the Board of Directors and shareholders in Regeneron Pharmaceuticals.

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Cell

How to Stop the Obesity Epidemic?

Ice Breaking

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China has entered the era of obesity. Data from China Noncommunicable Disease (NCD) Surveillance 2010 have shown that one in three Chinese adults had either central or general obesity. Meanwhile, the epidemic of childhood obesity may weigh on China's future. Obesity affects virtually all ages and socioeconomic groups and significantly contributes to the rocket-rising incidence of NCDs, including type 2 diabetes, cardiovascular diseases, and certain forms of cancer, which is worrisome for a country with a population of 1.37 billion.

The huge demographic pressure, unbalanced economic development, unmet social diversity, and childhood obesity epidemic have all created tough challenges for the Chinese government to fight against obesity. Prevention and control strategies must be comprehensive and should include proactive approaches: reducing health disparity through healthcare reform and development, protecting parental and childhood health, enhancing education on a healthy lifestyle, implementing awareness and detection programs for genetically susceptible individuals, and early interventions targeting high-risk population. To efficiently halt the obesity epidemic, the main focus should be placed on children and adolescents. The national research supporting systems should encourage biomedical scientists to explore the pathogenesis of obesity and develop safe and effective novel anti-obesity drugs/procedures toward gut microbes, brown fat, and genetic targets in regulatory network of metabolism. International cooperation is of key importance in basic research and translational studies. It is tough, but with hope.

Move It and Lose It

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There is a growing health burden arising from the interrelated sequelae of metabolic disorders comprising impaired glucose tolerance, type 2 diabetes, and sarcopenia. Obesity and physical inactivity are the main drivers of these metabolic disorders, with the risk of co-morbidities including hypertension, dyslipidemia, cardiovascular disease, stroke, cancer, sleep apnea, gallbladder disease, hyperuricemia and gout, and osteoarthritis. A critical health issue facing overweight adults is how to lose fat mass and improve whole-body glucose tolerance, while simultaneously preserving skeletal muscle mass. This goal is made especially difficult in the face of reduced levels of physical activity and increased longevity.

Modifiable lifestyle factors such as exercise training and diet are clinically proven, cost-effective, primary interventions that delay and, in many cases, prevent the health burdens associated with obesity. Yet, achieving this is easier said than done, and inertia is difficult to overcome. The principal challenge is to find "practical, ready-to-use" solutions to combat the growing epidemic-like increase in metabolic disease. This could be through the development of time-efficient, lifelong exercise intervention strategies that include dietary modifications. Importantly, these modifications must be readily incorporated into an individual's "everyday routine." A second goal is to provide clinical insight into the heterogeneity underlying not only the development of metabolic disease, but also individual differences in the response to treatment regimes. Given the widespread benefits of regular physical activity, it may be better to be fit and fat than lean and lethargic.

Research, Not Surgery

C. Ronald Kahn
Joslin Diabetes Center

As a physician-researcher in the field of diabetes and obesity, I am struck each year by the statistics showing the increasing prevalence of overweight and obesity and one of its major results, type 2 diabetes, with almost a million more cases each year in the U.S. alone. At the same time, I am struck by the great advances in research on cellular/molecular mechanisms underlying the control of energy balance through regulation of appetite and energy expenditure. The data are clear that the driving force in this epidemic is increasing levels of energy intake (168–335 kcal/day between 1970 and 2000), coupled with decreasing energy expenditure due to sedentary lifestyle. As one of my obese patients said, "Doc, I am digging my grave with my mouth." How are we going to change this trajectory? History has shown that changing behavior is difficult. Bariatric surgery works, but even in the best centers, surgical mortality rates are about 1 per 1,000—a level higher than we would ever accept for a medical therapy. So we have to find ways to convert our research into practical solutions. There are at least three areas of real hope: (1) unleashing the power of anorexigenic hormones, including hypothalamic, adipose-derived, and gut hormones; (2) stoking the fire of energy expenditure through agents that activate or increase the mass of brown/beige fat; and (3) finding the composition of gut microbiota that minimizes the impact of caloric excess on weight gain, insulin resistance, and metabolic dysfunction. With these, we can begin to stem the rising tide of obesity and its associated metabolic complications.

Fat Is Not Your Enemy!

Bruce Spiegelman
Harvard Medical School

Obesity is defined as a condition of excessive fat mass, but what do the fat cells do in normal physiology and in pathological states like obesity? White adipose tissue (ordinary fat) represents the major site for storage of chemical energy in mammals. When there is an imbalance between energy intake (eating) and energy expenditure (exercise, normal cellular processes, and thermogenesis), most of that excess energy is stored as triglycerides in fat. This is the proper and healthy place for energy storage, as lipid deposition in non-adipose tissues such as liver or muscle can impair their function. This occurs with adipose cell dysfunction (lipodystrophy) or when the excess energy simply overtakes the capacity of the fat to store those calories, as in many obese humans. A critical idea that emerged in the 1980s–90s was that adipose tissues are a critical "information hub" and signal metabolic status to the rest of the body through secretion of "adipokines," such as TNF-α, leptin, adipsin, and adiponectin. These affect insulin sensitivity, feeding behavior, and β cell function.

Recently, much attention has been focused on brown and beige adipocytes, thermogenic cells that exist in both rodents and humans. These cells dissipate chemical energy in the form of heat via uncoupled respiration. Increases in amounts of brown and beige fat have been shown to protect against obesity and diabetes in rodent models; strenuous efforts are now being undertaken to learn how to expand or activate these depots in ways that might be therapeutic in humans.

The Secrets of Outliers

Stephen O'Rahilly
University of Cambridge

Successful efforts in obesity prevention will require major changes to the obesogenic environment that will be easier to enact in some societies than others for cultural and political reasons. Even with such changes, however, there will still be some individuals who are susceptible to morbid obesity and others who develop catastrophic metabolic decompensation. To treat these people, we will need improved medicines. A foothold toward improved therapeutics has come from studies of human outliers who carry highly penetrant mutations. These studies have been enormously helpful in providing a "wiring diagram" for how processes such as energy balance or the maintenance of insulin sensitivity are regulated in humans. In metabolic disease, there are powerful recent examples of how the discovery of the causative genetic defect in very rare outliers for phenotypes such as bone density or serum cholesterol has directly led to the development of exciting therapeutics. In the area of obesity, leptin is a life-saving therapy for the rare children congenitally lacking the hormone and of great benefit to many more patients with lipodystrophy. We still lack comparable transformative therapies for commoner forms of obesity, however, which may in part be because many of the drug targets revealed by human genetics are in the brain, making them difficult to target. Nevertheless, I predict that human genetic studies of outliers for phenotypes such as extreme leanness and retention of normal insulin sensitivity despite massive obesity will be a fertile ground for the discovery of new targets of great therapeutic promise.

Brain behind Feeding

Jens C. Brüning
Max Planck Institute for Metabolism Research

Over the last 20 years, the field of obesity research has been revolutionized through the identification of important molecular pathways controlling energy homeostasis. This was pioneered by the identification of leptin as a fuel sensor providing feedback information to the CNS about energy availability in the periphery of the organism to adapt food intake and energy expenditure. This led to the identification of critical neurons that mediate the anorexigenic effects of leptin and that at the same time orchestrate multiple pathways in fuel homeostasis, including glucose metabolism. The recent developments in neurocircuitry mapping, including optogenetics, pharmacogenetics, and translational profiling of defined neurons, provide the basis for a complete understanding of the complex neurocircuitry controlling feeding, energy expenditure, and peripheral glucose metabolism, as well as the integration of these processes with higher cognitive functions. Defining the neuronal convergence points of these regulatory pathways and new modulators of this activity in my view holds the potential to develop a whole range of novel therapeutic targets for metabolic disorders. Moreover, having identified novel pathways involved in obesity through genome-wide association studies has revealed additional regulators of energy homeostasis. Defining their cellular and molecular actions will potentially open novel therapeutic routes as well. Collectively, we are facing an exciting era in obesity research with unprecedented promise for a deeper understanding of its pathophysiology and a plethora of unexpected therapeutic options.

176 Cell 161, March 26, 2015 ©2015 Elsevier Inc. DOI http://dx.doi.org/10.1016/j.cell.2015.03.014

SnapShot: GI Tract Development

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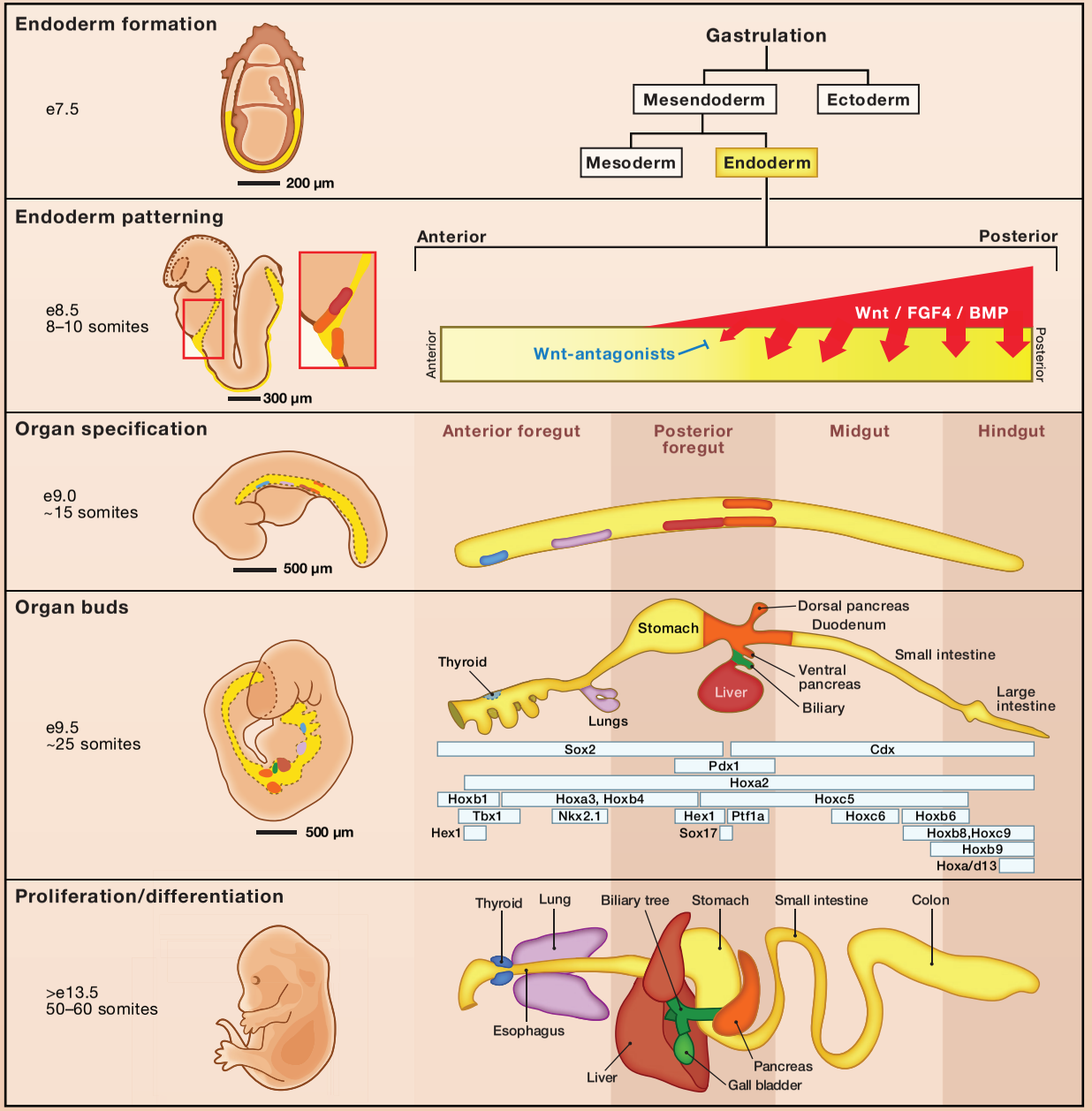
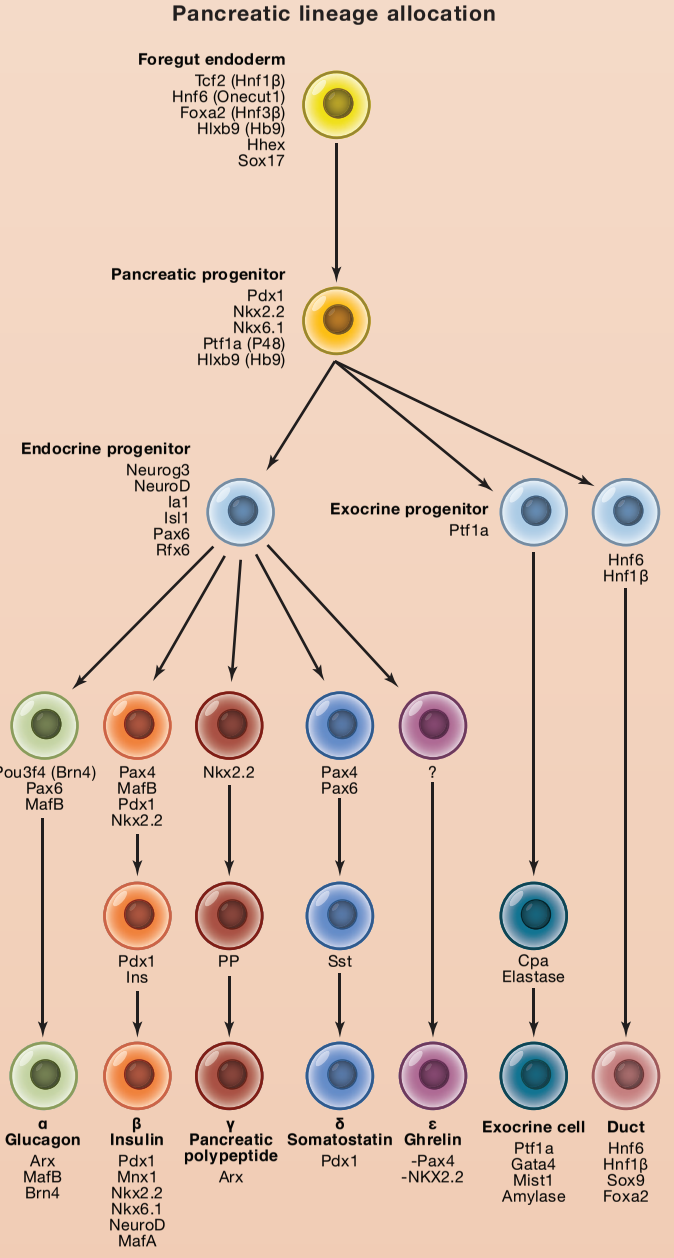
Cell





See online version for legend and references.

SnapShot: GI Tract Development

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Cell

The endoderm germ layer contributes to the respiratory and gastrointestinal (GI) lineages during development, giving rise to an array of specialized epithelial cell types lining organs, including the thyroid, thymus, lungs, liver, biliary system, pancreas, and intestines. This SnapShot timelines and summarizes key stages following gastrulation, including endoderm patterning, organ specification, and organogenesis. A lineage tree of the developing endocrine pancreas is outlined to further illustrate this process.

Timeline of Endoderm Formation, Patterning, and Organogenesis

During development in mice (left), the blastula gives rise to the three germ layers (ectoderm, mesoderm, and definitive endoderm) through the process of gastrulation (middle), which occurs between embryonic day 5 and 7.5 (e5–e7.5). After gastrulation, the two-dimensional sheet of definitive endoderm is patterned along the anterior-posterior (A-P) axis and undergoes morphogenesis to form a three-dimensional gut tube that is surrounded by a primitive mesenchyme (e8.5). A-P patterning of the endoderm occurs through reciprocal signaling with the mesenchyme involving growth factors such as Wnts, Fgfs, and Bmps. At this stage in development, these factors largely act to promote posterior fate and repress anterior fate. The anterior endoderm gives rise to the foregut (thyroid, lungs, esophagus, liver, stomach, pancreas), while the midgut and hindgut give rise to the small and large intestines, respectively. The first evidence of organ specification occurs in the early gut tube by the expression transcription factors that begin to demarcate specific organ domains, including the respiratory tract (Nkx2.1), liver (Hhex), stomach (Sox2 and Pdx1), extrahepatic biliary system (Sox17), pancreas (Pdx1 and Ptf1a), duodenum (Pdx1 and Cdx2), and intestine (Cdx2). The spatially restricted expression of these transcription factors predicts where organs will begin to form starting around e9.5. By e13.5, the organs of the respiratory and GI tracts are formed and undergoing growth and differentiation into specialized lineages.

Pancreatic Lineage Allocation: A Transcription Factor Map

Temporal lineage formation of the pancreas involves the expression of unique sets of transcription factors that mark and often direct cell fate decisions (right). All developing cell lineages of the pancreas (acinar, duct, and endocrine) arise from the foregut endoderm, which expresses markers such as Foxa2, Hnf6, and Hlxb9. The pancreatic endoderm becomes specified when the gut tube begins to express Pdx1 and Ptf1a in dorsal and ventral domains of the tube (e8.5–9.0). Morphogenesis of the pancreas initiates with an endodermal thickening (e9.0) and evagination of dorsal and ventral pancreatic buds (e9.5–e10.0) into the surrounding mesenchyme, forming an expanding pool of multipotent pancreatic progenitor cells. The lineage allocation and maturation of specific pancreatic cell subtypes are mediated by a network of signaling pathways and transcription factors. Commitment of progenitor cells to the endocrine lineage occurs following transient expression of Neurog3 and its downstream targets Neurod, Rfx6, and Pax6, whereas exocrine-committed cells express high levels of Ptf1a and carboxypeptidase A (CpA). Allocation of the separate endocrine lineages involves the combinatorial actions of multiple transcription factors. For example, development of mature β cells requires Pdx1, NeuroD, Nx6.1, and MafA. The ductal lineage involves a different set of factors, including Hnf1β and Hnf6. Each pancreatic cell lineage is portrayed with a subset of defining transcription factors throughout development.

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